Amelioration of Zidovudine-Induced Fetal Toxicity in Pregnant Mice

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The effects of zidovudine (AZT) on the fetus were investigated in pregnant mice by using parameters such as the number of fetuses, fetal size, and the fetal hepatic cell clonogenic assay. AZT caused dose-dependent toxicity to the fetus upon administration via drinking water to pregnant mice from days 1 to 13 of gestation. At the 0.5-mg/ml dose level, AZT caused a decrease in the number of fetuses to 12 from an average of 16.5 in control animals, and the fetal size (crown-rump length) was reduced from 10.5 to 8.5 mm. The CFU of the erythroid progenitor cell colonies derived from the fetal hepatic cells were decreased to 38% of that of the control, and the hematocrit dropped to 33.5 ± 1.7 from a control value of 42.6 ± 2.5. Concomitant administration of erythropoietin, vitamin E, or interleukin-3 to the AZT-treated pregnant mice caused a significant reversal in the AZT-induced toxicity to the fetus and to the mother's bone marrow. The success of therapeutic intervention was demonstrated by (i) restoration of the number of fetuses to the level of untreated controls, (ii) an increase in the size of fetuses to normal values, and (iii) an increase in hematocrit to >40. The results suggest that AZT is toxic to the fetus in a dose-dependent manner and that treatment with erythropoietin, vitamin E, or interleukin-3 can ameliorate the AZT-induced fetal toxicity.

Significant dose-related toxicity, primarily anemia and leukopenia associated with the administration of zidovudine (AZT), remains a limiting factor in the clinical management of AIDS (13, 20). With an increasing recognition of the heterosexual spread of human immunodeficiency virus (HIV) and a high rate of reproduction reported for HIV-positive women (79% in the reproductive ages), the importance of treating pregnant women with AIDS with anti-HIV drugs is apparent. A recent prospective study of infants born to HIV-seropositive mothers has concluded that approximately one-third of them will have evidence of HIV type 1 infection or AIDS by the age of 18 months (4). Furthermore, the perinatal transmission of HIV type 1 exerts a major adverse effect on the survival of the infant (21), indicating that effective antiretroviral therapy during gestation and in the perinatal period could potentially be very useful in the management of maternal transmission of HIV (25). Although AZT has been shown to cross the placental barrier and to achieve therapeutic levels in the body fluid of the infants during the first 36 h of life (7), there are relatively few published reports which describe the toxic effects of AZT on the embryo. An overview report by Ayers (3) indicated that there is no evidence of teratogenicity in the offspring of rats or rabbits given AZT during gestation; however, an increased incidence of late fetal resorption was observed. Another report demonstrated that administration of AZT to rats on day 10 of gestation had no effect on the growth or survival of the offspring (18). The effects of AZT on fetal growth in a pregnant mouse model were reported by our laboratory (15), in which we showed that AZT has a dose-dependent effect in reducing fetal size and number compared with the effects in untreated animals. Toltzis et al. (31) have also reported that administration of AZT to mice during gestation yields fewer fetuses and a greater number of resorptions per pregnant mouse compared with those in untreated animals. Those investigators further demonstrated that AZT produces a direct toxic effect on the mouse embryo in vitro, as determined by inhibition of development of the blastocyst stage from single-cell oocytes fertilized in vitro.

Although a recent clinical report (27) indicated that AZT is well tolerated by pregnant women, 3 of the 7 newborns with hemoglobin levels of less than 8.4 mmol/liter were born prematurely, and two cases of intraterine growth retardation were also observed among 38 infants delivered at term by women treated with AZT (27). Since fetal liver is deficient in glucuronyltransferase activity (the major enzyme used for the metabolism of AZT), the developing fetus would be exposed to a continuous high concentration of AZT, thus increasing the risk of toxicity. We have previously reported that AZT and other anti-HIV drugs, such as 2',3'-dideoxythymidine, 2',3'-dideoxy-2',3'-dihydrothymidine, and ribavirin, are significantly more toxic to murine fetal hepatic cells than to the bone marrow cells in vitro, as measured by the assay that measures the colony-forming ability of erythroid progenitor cells (14). We therefore chose to monitor AZT-induced fetal hepatic cell toxicity in pregnant mice and to investigate the role of erythropoietin (Epo), vitamin E, and interleukin-3 (IL-3) as candidates that may offer protection from this toxicity.

MATERIALS AND METHODS

Animal mating and maintenance. CD-1 mice (age, 80 to 90 days; Charles River, Wilmington, Mass.) were given food and water ad libitum. For mating, one male and three female mice were housed together for one night, and the appearance of copulatory plugs in the vaginas of the three females was monitored on the morning of the next day. The day after mating was called day 0 of gestation.

Drug administration. AZT was synthesized in our laboratory by a published procedure (2). The synthetic material was analytically pure, as determined by high-pressure liquid chromatography, UV spectrophotometry, and melting point.

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The recombinant human Epo was purchased from Genzyme Corporation (Boston, Mass.). IL-3 was a gift from Sandoz Corporation (East Hanover, N.J.), and vitamin E was purchased from Sigma Chemical Co. (St. Louis, Mo.). Mated mice were divided into groups of 5 to 10 mice each and were treated during days 1 to 13 of gestation, as follows. (i) The control group received no treatment. (ii) The AZT-treated group initially received a dose of 1 mg/ml administered via drinking water; approximately 60% of this group of animals had fetal resorption. Therefore, in subsequent experiments the dosage of AZT was reduced to one-half of the initial level (0.5 mg/ml). Epo, vitamin E, and IL-3 were administered at levels of 0.4 U per mouse per day given subcutaneously, 50 mg/kg of body weight per day given orally, and 100 U per mouse per day given subcutaneously, respectively. The other treatment groups were as follows: (iii) AZT, lower dose only; (iv) Epo only; (v) vitamin E only; (vi) IL-3 only; (vii) AZT plus Epo; (viii) AZT plus vitamin E, and (ix) AZT plus IL-3.

Number and size of fetuses. The number and size of fetuses per mouse were determined on day 13 of gestation in each group of pregnant animals described above. The dams were anesthetized with ether, and the uterine horns were removed and cut open in a petri dish. The number of fetuses in each animal within a group are counted and are reported as an average number. Fetal size (crown-rump length) was measured with vernier calipers after removing each fetus from their amniotic sacs. The data are expressed as a mean of the fetal size obtained from all the animals within each group.

Isolation of FHCs and BMCs. The hepatic tissue from each fetus was removed aseptically, pooled together for each dam, and minced; and cell suspensions were made in alpha medium to a concentration of 10⁶ cells per ml as described previously (14). On day 13 of gestation, the fetal hepatic cells (FHCs) in mice are primarily erythroid progenitor cells and have been shown to be exquisitely sensitive to Epo (29). The bone marrow cells (BMCs) were obtained from the same animals by aseptically removing the femurs and flushing the marrow with a 5/8-inch (1.6-cm) 25-gauge needle. The BMC suspension was made in RPMI 1640 medium to give a final concentration of 10⁶ cells per ml, according to the published procedure (16).

 Colony-forming ability of erythroid progenitor cells. The number of CFU of erythroid progenitor cells (CFU-E) was determined both in the BMCs obtained from the pregnant mice and in the FHCs isolated from the hepatic tissue of the fetuses on day 13 of gestation. Methylcellulose solution (1.3%; vol/vol) was prepared as described previously (16) in RPMI 1640 medium containing L-glutamine (2.0 mM), heat-inactivated fetal bovine serum (20%), 2-mercaptoethanol (0.1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Epo (200 mU/ml for BMCs or 50 mU/ml for FHCs) was added. The BMCs or FHCs (10⁶ cells per ml) were diluted 1:10 in the methylcellulose mixture. One milliliter of the cell suspension (10⁶ cells per ml) was plated in duplicate in 35-mm-diameter petri dishes, and the dishes were incubated in 5% CO₂ and 95% humidified air at 37°C for 48 h. The plates were stained with 3,3′-diaminobenzidine, and colonies containing eight or more benzidine-positive cells were counted under an inverted microscope (Olympus, Tokyo, Japan).

 Colony-forming ability of granulocyte-macrophage progenitor cells. For the assay that determined the number of CFU of granulocyte-macrophage progenitor cells (CFU-GM), methylcellulose solution (2%; vol/vol) was prepared as described previously (16) in RPMI 1640 medium containing L-glutamine (2.0 mM), fetal bovine serum (15%), 2-mercaptoethanol (0.1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and murine spleen cell conditioned medium (1%; Terry Fox Laboratories, Vancouver, Canada). One milliliter of cell suspension (10⁷ cells per ml) was plated and was incubated for 7 days as described above. Colonies containing 50 or more cells were counted.

Hematocrit. Blood samples were drawn from the anesthetized pregnant mice on day 13 of gestation by cardiac puncture with a syringe containing a 5/8-inch (1.6-cm), 25-gauge needle before removing the fetus and the maternal bone marrow. The isolated blood was filled in duplicate in microhematocrit capillary tubes. These tubes were spun for 3 min in an autocrit centrifuge (Clay-Adams, Inc., New York, N.Y.), and the values of hematocrit were determined on an autocrit centrifuge reader.

Data analysis. Statistical analyses were done by two-tailed Student t test. The drug-treated groups were compared with untreated controls at the P < 0.05 level of significance.

RESULTS

In the study described here, the fetal toxicity of AZT administered to pregnant mice was determined by measurement of fetal size, number of fetuses, and clonogenic assays for CFU-E derived from the FHCs. The numbers of CFU-E and CFU-GM derived from BMCs were also determined simultaneously. Administration of AZT at 1.0 mg/ml in drinking water reduced the number of fetuses by 60%, indicating that significant fetal toxicity is induced by AZT at this dose level. Furthermore, evaluation of the remaining fetuses at day 13 demonstrated a significant decrease in the fetal size by approximately 50% compared with the sizes of the control fetuses (Table 1). Therefore, in subsequent experiments, pregnant mice were treated with a lower dose of AZT (0.5 mg/ml in drinking water), which also resulted in a reduction in the number of fetuses, albeit only by 10 to 20%. This dose is equivalent to a daily dose of approximately 70 mg/kg on the basis of an average consumption of 5 ml of water per mouse per day and has been used earlier by other investigators to demonstrate AZT-induced hematopoietic toxicity in mice (5). There was no significant alteration in the amount of water consumed by the pregnant mice during the treatment period. The number of fetuses per mouse were significantly decreased to 12.0 upon administration of AZT.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. of animals/group</th>
<th>No. of fetuses/mouse*</th>
<th>Size of fetus (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>10</td>
<td>16.5 ± 2.2</td>
<td>10.5 ± 0.8</td>
</tr>
<tr>
<td>AZT (0.5 mg/ml)</td>
<td>10</td>
<td>12.0 ± 1.5**</td>
<td>8.5 ± 0.6**</td>
</tr>
<tr>
<td>AZT (1.0 mg/ml)</td>
<td>6</td>
<td>8.5 ± 2.0*</td>
<td>6.0 ± 1.6*</td>
</tr>
<tr>
<td>Epo</td>
<td>6</td>
<td>16.0 ± 2.2</td>
<td>10.5 ± 0.8</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>6</td>
<td>16.5 ± 2.0</td>
<td>10.5 ± 0.9</td>
</tr>
<tr>
<td>IL-3</td>
<td>6</td>
<td>15.0 ± 1.0</td>
<td>11.6 ± 1.1</td>
</tr>
<tr>
<td>AZT (0.5 mg/ml) + Epo</td>
<td>5</td>
<td>16.2 ± 2.0</td>
<td>10.5 ± 1.2*</td>
</tr>
<tr>
<td>AZT (0.5 mg/ml) + vitamin E</td>
<td>5</td>
<td>16.5 ± 2.2</td>
<td>10.5 ± 1.0*</td>
</tr>
<tr>
<td>AZT (0.5 mg/ml) + IL-3</td>
<td>5</td>
<td>14.0 ± 2.0</td>
<td>10.2 ± 0.8*</td>
</tr>
</tbody>
</table>

* The drugs were administered to the pregnant mice for 13 consecutive days beginning from day of gestation, as described in the text.
** Average total number of fetuses per mouse in each group.
* Significant by the Student t test when compared with the control group (P < 0.05).
* Significant by the Student t test when compared with the AZT-treated (0.5 mg/ml) group (P < 0.05).


at the lower dose level (0.5 mg/ml; Table 1), from an average of 16.5 fetuses for the control mice. Fetal size was reduced to approximately 80% of the control value, from 10.5 to 8.5 mm. Administration of Epo, vitamin E, or IL-3 alone did not cause any significant change in the number of fetuses or fetal size compared with those in the untreated control group. Administration of Epo, vitamin E, or IL-3 to pregnant mice simultaneously treated with AZT protected the animals from the AZT-induced toxicity, as demonstrated by restoration of the number of fetuses to the level in untreated controls and an increase in the size of the fetuses to normal levels (Table 1).

The numbers of CFU-E (Fig. 1A) and CFU-GM (Fig. 2) derived from the BMCs of AZT-treated pregnant mice at day 13 of gestation were significantly decreased, to 47 and 57% of the numbers in controls, respectively, as expected. However, a similar effect was also observed with the number of CFU-E derived from FHCs, which was decreased to 38% of the level in controls (Fig. 1B). Administration of Epo, vitamin E, or IL-3 to the pregnant mice simultaneously receiving AZT caused a significant increase in the numbers of CFU-E derived from BMCs to 66, 63, or 89% of the number in controls, respectively, as compared to the AZT-treated group (Fig. 1A). Similarly, for mice given Epo, vitamin E, or IL-3, the number of CFU-E derived from FHCs also increased to 77, 84, or 71%, respectively, of the levels in controls (Fig. 1B). Treatment with any of the three agents, Epo, vitamin E, or IL-3, also increased the numbers of CFU-GM derived from the BMCs of AZT-treated animals to 86, 72, and 99%, respectively, of the numbers in controls (Fig. 2). The hematocrit in AZT-treated pregnant mice dropped to 33.5 ± 1.7 from a control value of 42.6 ± 2.5 (Fig. 3). Simultaneous treatment of these animals with Epo or vitamin E significantly increased the hematocrit to 40.6 ± 2.6 and 40.4 ± 1.5, respectively. However, the increase in the
hematocrit of the animals simultaneously treated with IL-3, although substantial, was not found to be significant. The hematocrit of AZT-untreated animals administered Epo, vitamin E, or IL-3 showed an increased trend, but it was not significantly greater than the hematocrit of the control group.

**DISCUSSION**

The data in this report demonstrate that AZT causes a pronounced and dose-dependent toxic effect on the development of fetuses in pregnant mice, in addition to its well-known toxicity observed in bone marrow. The mice in the experiments described here were not treated with AZT prior to mating, thus minimizing the effect of chronic maternal toxicity of AZT on fetal growth. This is in contrast to a protocol of AZT therapy for 6 weeks before mating, which led to a considerable number of pregnancy failures (31). Since AZT is significantly more toxic to FHCs (which are primarily erythroid in the early gestation period) than the BMCs (14), it is likely that the fetal toxicity observed in the experiments described here is a direct effect of AZT. These results are consistent with an earlier report showing that AZT has a direct toxic effect on the developing mouse embryo (31). However, an indirect effect caused by AZT-induced anemia may also have a significant role on fetal growth, specifically during the mid to late periods of gestation. Important interactions have been indicated to exist between AZT-induced hematopoietic toxicity and the endocrine system, causing subtle and complex effects of AZT on the reproductive system in male rats (24). It is therefore likely that such indirect effects may also contribute to the observed AZT-induced fetal toxicity.

Three agents, Epo, vitamin E, and IL-3, were used in the study to determine whether simultaneous administration of any one of these agents can overcome the toxic effects of AZT on the fetal tissue. Recombinant human Epo has been recently approved by the U.S. Food and Drug Administration for treatment of AZT-induced anemia in patients with AIDS. However, the beneficial response to Epo was limited to those patients whose endogenous Epo was ≤500 IU/liter (12). Since AZT therapy in patients with AIDS has been shown to produce elevated Epo levels in serum (30), it appears either that higher levels of Epo in serum may be necessary to ameliorate anemia or that the administration of Epo to these patients may be of limited value. Epo, however, has been shown to possess a burst-promoting activity and to act as a terminal differentiating hormone in the fetal liver (11). Therefore, it was deemed desirable to investigate whether Epo can ameliorate AZT-induced fetal toxicity.

Vitamin E has also been shown to cause an erythropoietic response in anemic patients (10, 28) and to restore cell-mediated immunity by increasing the T-cell number (8). Since vitamin E affects both the hematopoietic and immune systems, experiments were designed to evaluate the effects of combining vitamin E with AZT with respect to the ability of vitamin E to overcome AZT-induced hematopoietic toxicity. We have reported that d-α-tocopherol acid succinate, a relatively more soluble derivative of vitamin E, not only partially protected the bone marrow from AZT-induced toxicity under in vitro conditions but also increased the anti-HIV potency of AZT in MT-4 cells (16). We have further demonstrated that vitamin E is approximately equal to Epo in its ability to overcome the AZT-induced anemia in CD-1 male mice (17). Vitamin E has also been shown to penetrate the placental barrier, and its permeability remained relatively constant through advancing gestation (1).

IL-3, a hematopoietic colony-stimulating factor, has been shown to stimulate the growth of hematopoietic progenitors in vitro (17) and in vivo in preclinical animal models (9). Recently, recombinant human IL-3 has also been shown to induce a multilineage response by stimulating the proliferation of hematopoietic progenitor cells in cancer patients with preserved hematopoietic function and bone marrow failure (19). Furthermore, it has been reported that IL-3 does not interfere with the anti-HIV activity of AZT, although it stimulated HIV expression in monocytes (22). The data presented here indicate that fetal tissues are permeable to all the three agents, Epo, vitamin E, and IL-3, since the administration of these drugs to AZT-treated pregnant mice restored fetal development and stimulated the growth of FHCs. These data further demonstrate that erythroid progenitor cells derived from fetal liver or bone marrow are responsive not only to hematopoietic growth factors such as Epo or IL-3 but also to vitamin E. It is not obvious from the results of the studies, however, whether the beneficial effect of vitamin E is due to its direct stimulatory effect on the proliferation of FHCs and bone marrow progenitor cells or if it is mediated through an indirect release of a hematopoietic growth factor. Although it is likely that the protective effects of Epo, vitamin E, and IL-3 on fetal development from AZT-induced toxicity may be partially related to the reversal of the maternal anemia, our earlier in vitro data (14) suggest a direct role of these agents on FHC growth.

The mechanism of in vivo and in vitro AZT toxicity on FHCs may be expected to be similar to that of several possible biochemical mechanisms which have been suggested for AZT-induced toxicity to erythroid progenitor cells of the fetal bone marrow. Sommadossi et al. (26) have demonstrated a direct relationship between AZT incorporation into DNA and inhibition of formation of CFUs of erythroid progenitor cells after exposure of BMCs to various concentrations of AZT. Alternatively, AZT has also been shown to inhibit mitochondrial DNA replication in isolated rat mitochondria (25). It is not known whether the incorporation of AZT into the cellular DNA or the inhibition of replication of mitochondrial DNA is involved in AZT-induced fetal toxicity. It is unlikely that Epo, vitamin E, or IL-3 modulates the cellular enzymes in the progenitor cells, leading to the inhibition of formation of AZT triphosphate, the activated substrate needed for incorporation of AZT into DNA. Furthermore, it is not known whether phosphorylation of AZT in murine FHCs differs from that in human tissue. It appears from the results of our studies that the protective effect of Epo, vitamin E, or IL-3 against AZT-induced toxicity to FHCs and BMCs, as demonstrated by significant increases in CFU and hematocrit, is due to a dose-dependent proliferation of progenitor cells by the growth factors, thus overcoming the AZT-induced toxicity.

Although the mechanisms associated with the toxicity of AZT to the fetus are unknown, the results reported here suggest a dose-dependent toxicity of AZT to fetal cells. These data provide further evidence that the concomitant administration of Epo, vitamin E, or IL-3 may be of potential clinical benefit in overcoming the toxic effects of AZT in pregnant women with AIDS.

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