Effect of Zidovudine on Preimplantation Murine Embryos

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It previously has been demonstrated that zidovudine (AZT) is lethal to early murine embryos. The effect of the drug on pre- and postimplantation embryos was examined to delineate the timing of this toxicity and to investigate its possible mechanisms. Embryos exposed in the whole mouse during preblastocyst development were unable to proceed beyond the blastocyst stage. Similarly, when two-cell embryos harvested from unexposed females were exposed to low-concentration (1 μM) AZT in vitro over 24 h, development beyond the blastocyst stage was inhibited. In contrast, drug exposure during in vitro blastocyst and postblastocyst development resulted in little or no morphologic toxicity. Further investigation revealed that preblastocyst AZT exposure resulted in the development of blastocysts with significantly lower cell numbers than control embryos. While embryonic exposure to AZT at the blastocyst and postblastocyst stages also resulted in retarded cell division, the effects were milder than those recorded after preblastocyst exposure. These data demonstrate that the critical period of AZT toxicity toward murine embryos is between ovulation and implantation and indicate that AZT directly suppresses cell division in the preimplantation embryo.

As the incidence of human immunodeficiency virus (HIV) infection in women increases, growing numbers will be deliberately or inadvertently exposed to zidovudine (AZT) during pregnancy. Recommendations to treat HIV-infected pregnant women with AZT if their CD4 cell counts fall below 200/μl (11) have been made. Moreover, trials to test whether antiviral chemotherapy of all pregnant HIV-infected women can interrupt the transmission of virus from mother to child are under way. Traditionally, therapy during pregnancy with any drug has been approached with extreme caution because of concerns about untoward effects upon the embryo or fetus. Therefore, it is important to investigate the toxicity of intragastential administration of HIV anti-metabolites in order to enable rational application to humans. It previously has been demonstrated that AZT at concentrations that are readily achievable with conventional human dosing is lethal to murine embryos during early gestation (7, 21). Direct exposure of embryos to AZT in vitro resulted in retarded cell division at 0.1 μg/ml and cell death at concentrations of 1 μg/ml and above (21). Safe administration of AZT during human pregnancy requires that this toxicity be better defined and that its underlying mechanism be determined.

The first step in defining the embryo toxicity of AZT is to identify the developmental stage at which this effect occurs. Murine embryonic development is sufficiently accessible to in vitro experimental manipulation that all stages can be examined for their susceptibility to drug toxicity by previously established methods (8, 16). Fertilized murine oocytes can be readily isolated from the dam at the one- to two-cell stage. Two-cell embryos can be cultured in vitro and will develop into blastocysts, the stage immediately preceding implantation in vivo, over approximately 3 days. Isolated blastocysts can be induced in vitro to further spread their trophoderm, the placental precursor, and to develop a well-rounded inner cell mass over an additional 3 to 4 days, corresponding to events occurring immediately after implantation in vivo. The inner cell mass will continue to grow over an additional 2 to 3 days. The following experiments were conducted with this system to determine the timing of AZT effects on murine embryogenesis and to suggest possible mechanisms for this toxicity.

MATERIALS AND METHODS

In vivo AZT exposure of oocytes. The effect of AZT upon the developing oocyte was examined by exposing CF-1 dams housed five per cage (until superovulation) to AZT (the commercially available drug) in their drinking water at a concentration of 0.25 mg/l. This exposure previously has been shown to result in a mean concentration of 0.12 μg/ml (0.45 μM) in serum and to be associated with significant pregnancy failure (21). Control mice were examined in parallel with the treated animals and were handled identically except that they received water free of drug. Mice were exposed to AZT over the 3 weeks preceding coitus, the time required for follicular development in the mouse ovary. The dams were induced to superovulate by using standard methods (8), and copulation was confirmed by detection of a vaginal plug. AZT was discontinued 1 day prior to superovulation (to limit AZT exposure to follicular development) or was continued until the third day of pregnancy (to include the period of preimplantation development). Preimplantation blastocysts were harvested on day 3 postcoitus from the fallopian tubes and were placed individually in 60-well plates (Nunc Inc., Nateville, Ill.) containing Dulbecco modified Eagle (DME) medium adjusted to 10% fetal calf serum but containing no AZT, and further in vitro development was assessed by light microscopy as described below.

In vitro AZT exposure of preblastocyst embryos. Two-cell embryos from superovulated females which had not been exposed to AZT were recovered on day 1 postcoitus and cultivated to the blastocyst stage in Akiko's medium by standard methods (8). AZT was prepared and sterilized in a fashion previously described (21), and the effect of the drug upon the period of preblastocyst development was examined by adjusting the medium to 1 μM AZT on each of the 3 days.
required for blastocyst formation in vitro. After each 24-h exposure, the cells were washed serially in media containing no drug and were allowed to proceed to the blastocyst stage in the absence of AZT. In this manner, the effect of the drug upon two-cell (day 1), four-cell (day 2), and eight-cell-tomorula (day 3) embryos could be tested. Control cells were cultured in the absence of drug side by side with exposed cells and were treated identically but were not subjected to washing. Development to the blastocyst stage was judged successful from light-microscopic appearance (8, 16).

Embryos that had successfully reached the blastocyst stage after AZT exposure were allowed to develop further by washing them a second time and transferring them individually to 60-well plates containing DME medium adjusted to 10% fetal calf serum in the absence of drug. In vitro shedding of the zona pellucida ("hatching") and subsequent attachment and spreading of trophectoderm with growth of inner cell mass on top of the trophectoderm layer ("outgrowth") was achieved over an additional 3 to 4 days, as judged by light microscopy.

In vitro AZT exposure of blastocysts. To assess the effect of AZT upon hatching of blastocysts which had not been previously exposed to AZT, preimplantation blastocysts were harvested from superovulated mice on day 3 postcoitus and placed in DME medium adjusted to 10% fetal calf serum plus AZT at concentrations ranging from 0 to 200 μM over the entire 3 to 4 days required for further development. Hatching and outgrowth development were assessed microscopically as described above. To assess the effect of AZT upon embryos that had already successfully hatched and developed trophectoderm outgrowths, preimplantation blastocysts were harvested from superovulated mice and allowed to hatch and form outgrowths in the absence of drug. The medium then was adjusted to AZT concentrations ranging from 0 to 100 μM, and the inner cell mass was allowed to divide further over an additional 2 days. Drug effects were assessed by microscopic examination.

Determination of embryonic cell numbers. The effect of 24-h AZT exposure (0, 1, and 10 μM) on embryonic cell division was determined at the two-cell, morula, midhatching, and posthatching stages of development. Embryonic cell numbers were determined by cell lysis in lactic acid and Giemsa staining of the released nuclei, as described by Magnuson and Epstein (10).

RESULTS

AZT exposure of developing oocytes in the whole mouse does not alter early in vitro embryonic development. The following experiments were designed to examined the effects of AZT on successive stages of murine development, namely, the period of oocyte development, the period ranging from fertilization to implantation, and the period immediately following implantation. The effect of the drug upon the developing oocyte was examined in the whole animal to avoid the difficulties involved in in vitro isolation and manipulation of the immature egg. In vivo exposure of the fertilized oocyte through the first 3 days postcoitus resulted in significant inhibition of in vitro blastocyst hatching and outgrowth development when the embryos were harvested immediately prior to implantation (P = 0.008) (Table 1). In contrast, drug exposure limited to prevolution oocyte development resulted in no detectable effect on in vitro blastocyst hatching and outgrowth development (P = 0.322) (Table 1), suggesting that AZT embryonic toxicity was confined to the period after ovulation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice mated/total</th>
<th>No. of blastocysts successfully hatched/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>8/10</td>
<td>20/26 (77)</td>
</tr>
<tr>
<td>AZT prior to ovulation</td>
<td>6/10</td>
<td>35/53 (66)*</td>
</tr>
<tr>
<td>AZT through ovulation and early pregnancy</td>
<td>6/9</td>
<td>24/53 (45)*</td>
</tr>
</tbody>
</table>

* Mice were given AZT in water for 3 weeks. Half of the exposed cohort had AZT discontinued prior to superovulation and mating, and half had the drug continued through the first 3 days of pregnancy. Embryos were harvested 3 days after copulation and allowed to hatch in vitro in the absence of drug.

*b P = 0.322 compared with control values, by the chi-square test.
+c P = 0.008 compared with control values, by the chi-square test.

In vitro exposure of preblastocyst embryos inhibits outgrowth development. To confirm the in vivo findings, embryos were isolated from AZT-unexposed superovulated dams at the two-cell stage and were exposed in vitro to 1 μM AZT for a short duration (24 h) on either day 1, day 2, or day 3 of preblastocyst development; exposure to AZT at higher concentrations (≥10 μM) was nearly uniformly lethal. Short-term exposure to 1 μM AZT had little effect on in vitro embryonic development to the blastocyst stage (P > 0.05 for all samples; data not shown). However, when 24-h drug-exposed embryos which had successfully reached the blastocyst stage were allowed to develop further in the absence of AZT, postblastocyst growth was severely inhibited in all samples (Fig. 1; P < 0.001 compared with control values), mirroring the findings observed after whole-animal exposure. The majority of embryos which had been drug exposed during preblastocyst development demonstrated collapse of the blastocoel without hatching from the zona pellucida. Smaller numbers aborted during the middle of hatching or suffered poor development of the inner cell mass. These findings indicated that short-term AZT treatment between ovulation and blastocyst formation had a direct inhibitory effect on postblastocyst development.

In vitro AZT exposure of mouse embryos beyond the...
blastocyst stage has little morphologic effect. To determine whether morphologic toxicity of AZT occurred in embryos exposed beyond the blastocyst stage, blastocysts harvested from unexposed dams were exposed in vitro to increasing concentrations of AZT continuously over 3 additional days (Fig. 2). Although a dose-dependent inhibition of trophoderm outgrowth and inner cell mass formation was noted, relatively mild effects were measured even at drug concentrations of up to 200 μM. By contrast, when two-cell embryos were exposed continuously over 3 days to only 10 μM AZT, profound inhibition of development to the blastocyst stage (95%) was measured (Fig. 2). The effect of AZT upon embryos which had already hatched and developed trophoderm outgrowths was investigated by adding the drug after the embryos had reached this stage. Drug exposure over 2 to 3 additional days, the limit of viability for this tissue in vitro, revealed no morphologically apparent toxicity at concentrations up to 100 μM (data not shown).

Short-term exposure of embryos to AZT results in retarded cell division. Although 24-h exposure of preblastocysts appeared to have no morphologic effect on development to the blastocyst stage, it was possible that resultant blastocysts had reduced cell numbers. This possibility was suggested by our own previous experiments indicating that low-concentration AZT exposure slows embryonic cell division without producing overt cytotoxicity (21), coupled with prior observations indicating that blastocysts containing diminished cell numbers fail to survive beyond implantation (19, 20). Embryos which had been harvested at the two-cell stage were exposed on either day 1 or day 3 to 1 μM AZT and allowed to develop to the blastocyst stage, and blastomere nuclei were stained and counted. Drug exposure resulted in significant depression of cell numbers in developed blastocysts. Unexposed embryos contained 57.50 ± 15.63 cells per blastocyst, in comparison with embryos which had been exposed to AZT at the two-cell stage (37.80 ± 9.39 cells per blastocyst, P < 0.001 compared with control values) and at the eight-cell-to-morula stage (38.47 ± 13.57 cells per blastocyst, P < 0.001 compared with control values). These results indicated that AZT exposure resulted in inhibition of cell division in the early murine embryo.

Since preblastocyst exposure resulted in diminished cell numbers without producing overt morphologic toxicity, cell numbers of drug-exposed postblastocyst embryos were measured to determine whether a corresponding effect occurred at that stage as well. As in the prior morphologic studies, exposure at the blastocyst and postblastocyst stages resulted in much milder toxicity than exposure at the preblastocyst stage. Embryos exposed for 24 h to AZT at midhatching and after hatching and outgrowth were completely demonstrated a dose-dependent inhibition of cell division (Table 2). However, this trend was not statistically significant, and it occurred at drug concentrations more than 10-fold higher than that required to produce similar inhibition in two-cell embryos.

**DISCUSSION**

An increasing number of women of childbearing age are being placed on antiretroviral chemotherapy as HIV infection spreads through nonhomosexual means. Recently, examination of 43 infants who had been exposed to AZT during pregnancy revealed no teratogenic effects, even when fetal exposure occurred during the first trimester (17). These findings mirrored those previously noted in retrovirally infected mice which had been exposed to the drug through their drinking water during middle to late gestation; no adverse drug effects were detected in the offspring (13, 14). We and others have found, however, that AZT results in death of mouse embryos when exposure occurs in the first half of gestation (7, 21). Our experiments presented here demonstrated that the critical period of AZT toxicity toward mouse embryos is between ovulation and implantation.

The present experiments were performed to provide a preliminary rationale for administration of AZT in pregnant humans and to suggest possible mechanisms of toxicity. The system that was employed, namely, the exposure of preblastocyst mouse embryos to potentially toxic substances, has been used to predict the adverse effects of a variety of physical and pharmacologic agents in humans (16). The correlation with human toxicity is variable: cyclophosphamide exposure and chlorambucil exposure, for example, which, similarly to AZT exposure, result in diminution of cell numbers in preimplantation murine embryos, are clearly associated with human risk, while chlorpromazine exposure, which results in a similar phenomenon in mouse blastocysts, has less certain effects during human gestation (2). Although caution must be exercised in extrapolating our findings to human pregnancy, the critical period of AZT toxicity should be completed by the middle of the first trimester. However, the current experiments further document that exposure of postblastocyst embryos to AZT at pharmacologically rele-

**TABLE 2. Effect of AZT exposure on postblastocyst cell division**

<table>
<thead>
<tr>
<th>Stage of growth</th>
<th>AZT dose (μM)</th>
<th>n</th>
<th>No. of cells (mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midhatching</td>
<td>0</td>
<td>20</td>
<td>89.35 ± 50.06</td>
<td>0.819</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>19</td>
<td>86.13 ± 39.38</td>
<td>0.532</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18</td>
<td>80.66 ± 29.56</td>
<td></td>
</tr>
<tr>
<td>Posthatching</td>
<td>0</td>
<td>8</td>
<td>277.00 ± 126.47</td>
<td>0.614</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6</td>
<td>222.83 ± 83.76</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>174.75 ± 83.90</td>
<td></td>
</tr>
</tbody>
</table>

*Embryos were exposed to AZT in vitro for 24 h, lysed, and stained. Cell numbers of AZT-exposed embryos were compared with those of unexposed embryos by Student's t test.*
vant concentrations mildly retards cell division. Other investiga-
tors have recently established that AZT exposure during the first half of murine pregnancy results not only in fewer fetuses per mouse but in significantly diminished crown-to-
rump lengths among the fetuses which survive (7). This growth retardation may have resulted from depressed cell division, as found in the current report, or from fetal hepatic and bone marrow cell toxicity, as suggested by others (7).

Some mechanisms of embryonic toxicity are suggested by the current experiments. Equivalent degrees of AZT-related inhibition were noted in embryos exposed at the two-cell or eight-cell-to-morula stage, suggesting that exposure to the drug at any time prior to blastocyst formation resulted in an immediate inhibition of cleavage. This observation is most consistent with an enzyme-inhibitory effect which reverses rapidly once the embryo is removed from the drug, although AZT has little inhibitory effect upon cellular DNA poly-
merase alpha (22). Inhibition of cleavage in the developing preblastocyst by AZT in itself may have prohibited develop-
ment beyond the blastocyst stage, since a critical number of inner cell mass cells in the blastocyst is required for further development (19, 20). Although inhibition of cell division was noted even in embryos treated after blastocyst hatching and outgrowth, the effects were considerably milder and may have occurred at a time when cell number was not critical for continued embryonic survival.

It is also possible that drug exposure may result in subcellular toxicities affecting embryonic development in a more indirect manner. It has been suggested recently that the primary cellular target of AZT and other dideoxynucleo-
sides drugs is the mitochondrion (1, 4, 6, 9, 12, 15). DNA polymerase gamma, the enzyme responsible for replication of mitochondrial DNA, is severely inhibited by these com-
pounds in cell-free assays (1, 4, 5, 9, 15, 22). These observa-
tions may be particularly relevant to the embryo lethality noted with AZT, since the period of maximal cytotoxicity seen in embryos (between ovulation and blastocyst forma-
tion) closely coincides with a period of mitochondrial mor-
phologic evolution in the mouse embryo (3, 18). The mor-
phologic maturation of the mitochondria at the blastocyst stage may correspond to the disappearing sensitivity of the embryo to the lethal effects of the drug once implantation has occurred.

The experiments presented here have documented that AZT is most toxic to murine embryos at the stage between ovulation and implantation. Effects on cell division persist after blastocyst hatching but are not morphologically appar-
ent and occur only at concentrations 10-fold higher than those associated with preblastocyst toxicity. These data suggest an inhibitory effect upon an embryonic cellular polymerase, a morphologically immature mitochondrion, or both.

ACKNOWLEDGMENTS

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