Immunomodulatory Effect of Zidovudine (ZDV) on Cytotoxic T Lymphocytes Previously Exposed to ZDV

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In a previous study, zidovudine (ZDV) was shown to cause a concentration-dependent inhibition of antigen-specific cytotoxic T-lymphocyte (CTL) clonal expansion (S. Francke, C. G. Orosz, K. A. Hayes, and L. E. Mathes, Antimicrob. Agents Chemother. 44:1900–1905, 2000). However, this suppressive effect was lost if exposure to ZDV was delayed for 24 to 48 h during the antigen sensitization period, suggesting that antigen-primed CTL may be less susceptible than naive T lymphocytes to the suppressive effects of ZDV. The present study was undertaken to determine if naive T lymphocytes were more sensitive to the suppressive effects of ZDV than T lymphocytes previously exposed to antigen. The 50% inhibitory concentration (IC₅₀) values of ZDV were determined on naive and antigen-primed T-cell responses in an alloantigen system. Lymphocyte cultures with continuous antigen exposure (double prime) were more resistant to ZDV suppression (IC₅₀ = 316 μM) than were naive lymphocytes (IC₅₀ = 87.5 μM). Interestingly, lymphocytes that were antigen primed but deprived of antigen during the final 7 days of culture (prime/hold) were equally sensitive to ZDV suppression (IC₅₀ = 29.3 μM). The addition of 80 μM ZDV during the initial priming of the single-prime (prime/hold) and double-prime cultures did not select for a more drug-resistant cell population. The differences in ZDV sensitivities are likely a reflection of the physiological properties of the lymphocytes related to their activation state.

Zidovudine (azidothymidine; ZDV), in single or combination therapy, is one of the major drugs used to treat AIDS. However, its positive therapeutic value is countered by clinical side effects and the development of drug resistance (4, 16, 17, 27, 37). ZDV also has potentially troubling properties affecting immune cell functions, such as suppression of antigen-driven T-cell proliferation (18), prolongation of the cell cycle (10, 49), and inhibition of a number of other immunologic responses including lectin- and antigen-induced mitosis, mixed lymphocyte culture reactions, and induction of the cytotoxic T-lymphocyte (CTL) response (18, 26, 36, 45). Mechanisms by which ZDV influences cell, and specifically immune cell, physiology are largely unknown, and the biologic relevance of these effects in vivo is speculative. However, the potential impact of ZDV on CTL-mediated cytolysis is of particular concern given the importance of these cells in combating human immunodeficiency virus (HIV) infection (25, 30, 46, 50).

In a previous study that used limiting dilution analysis, we reported that ZDV caused concentration-dependent inhibition of clonal expansion of antigen-specific CTL, suggesting that the basis for ZDV-related immunosuppression is stasis of T-cell expansion (18). In this study the estimated frequency of alloantigen-specific CTLs was profoundly lower in in vitro sensitization assays, where ZDV was present during primary antigen exposure (18). However, this suppressive effect was lost if exposure to ZDV was delayed for 24 to 48 h during the antigen sensitization period. The results suggested that antigen-primed CTL may be less susceptible to the suppressive effects of ZDV than naive T lymphocytes.

The objectives of the present study were (i) to measure the sensitivity of naive T lymphocytes to the suppressive effects of ZDV, and (ii) to determine if T cells sensitized to antigen in the presence of ZDV generated CTL with greater resistance to ZDV suppression. In order to explore this possibility, we used a 50% inhibitory concentration (IC₅₀) assay to measure the relative suppressive effect of ZDV on naive and primed CTL. The results suggest that naive cytotoxic T cells are two to five times more sensitive to the inhibitory effects of ZDV than are activated antigen-primed cells. However, previously antigen-primed T cells that were cultured without antigen but given interleukin-2 (IL-2) were shown to have increased sensitivity to ZDV over that determined for naive CTL. Antigen priming in the presence of ZDV did not generate a cytotoxic T-cell population with greater resistance to ZDV suppression.

MATERIALS AND METHODS

Mice. Animal studies were performed in accordance with the University Laboratory Animal Care and Use Committee and DHEW publication no. NIH 74-23, Guide for the Care and Use of Laboratory Animals. Six- to 8-week-old female DBA/2 (H-2b, Mls⁻) and C57BL/6 (H-2b, Mls⁻) mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, Ind.). The mice were housed in a laminar flow cabinet (animal storage isolator; Nu Aire Inc., Plymouth, Minn.) in groups of 5 to 10 animals per cage. Upon arrival, all mice were allowed a 7-day period of acclimation before use. Animals were sacrificed and spleens were collected within 2 weeks following the acclimation period.

ZDV. ZDV was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), as a lyophilized powder and stored light-protected at room temperature. ZDV was dissolved in cell culture medium for in vitro studies.

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Cell harvest, separation, and culture. Intact spleens from mice euthanized in a standard CO₂ chamber were dispersed into single-cell suspensions of splenocytes and washed three times in sterile phosphate-buffered saline. Tissue culture medium was Dulbecco's modified Eagle's medium supplemented with 1.6 mM L-glutamine, 0.27 mM folic acid, 0.27 mM L-asparagine, 0.55 mM L-arginine, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 U of penicillin-streptomycin/ml (Gibco, Grand Island, N.Y.), 5 × 10⁻⁵ M β-mercaptoethanol (Sigma Chemical, St. Louis, Mo.), and 10% heat-inactivated fetal calf serum, with 10 IU of IL-2/ml (Boehringer Mannheim) freshly added.

Study design. (i) Animal model. The experimental model for testing T-cell sensitivity to ZDV is based on the one-way mixed lymphocyte response (MLR) between the major histocompatibility complex-distinct mouse strains C57BL6 (responder) and DBA (stimulator). The product of the MLR is a C57BL6 T-cell population of T cells: naive (fresh splenocytes from C57BL6 mice), single primed (prime/hold), or double primed.

The rationale for using prime/hold and double-prime CTLs is as follows: alloantigen-activated CTL, in contrast to nonactivated CTL precursors, no longer require contact with specific alloantigens to allow lymphokine-mediated clonal expansion and subsequent detection in microcultures (43). Exposure to a single round of allogeneic antigen stimulation, however, leads to activation of only a fraction of all antigen-specific cells. Subsequent reexposure to the same antigen (double priming) increases the percentage of antigen-reactive cells by two mechanisms: (i) clonal expansion of already antigen sensitized cells, and (ii) first-time activation of cells which did not respond to the antigen during the first encounter.

(ii) Experimental design. The experimental design had five arms (Fig. 1).

(a) Arm 1 (naive CTL). Naive CTL were fresh splenocytes from C57BL6 mice placed directly into the IC₅₀ assay. Briefly, 3 × 10⁵ C57BL6 splenocytes/well (responder cells) were incubated for 7 days with 3 × 10⁵ irrDBA-2 splenocytes/well (sensitizing cells) in U-bottom 96-well plates in the presence of ZDV concentrations ranging between 0 and 1,000 μM. Each ZDV concentration was tested in replicates of six.

(b) Arm 2 and Arm 4 (double prime). For the double-primed CTL cultures, 10⁵ C57BL6 splenocytes were incubated with 10⁵ irrDBA-2 splenocytes for a period of 14 days at 37°C in 10% CO₂ in the presence of IL-2/ml (Boehringer Mannheim) freshly added.

(c) Arm 3 and Arm 5 (prime and hold). The prime/hold cultures were the same as the double-prime cultures during the initial 14-day culture period (Fig. 1). At that point, however, instead of adding irradiated DBA cells, these cultures received 10⁵ irradiated C57BL6 splenocytes/well as feeder cells in preparation for the IC₅₀ assay. Arm 5 contained 80 μM ZDV during the initial 14-day culture, while Arm 3 was grown without ZDV (Fig. 1).

The IC₅₀ assay. The IC₅₀ assay followed the procedure outlined in Fig. 1. As described above, the C57BL6/irrDBA-2 allogeneic mouse system was used as a source of CTL to measure the in vitro effect of ZDV on CTL effector cells. The assay procedure was adapted from a previously described protocol for determining precursor frequency by limiting dilution analysis (42, 43). The assay measures drug-mediated inhibition of in vitro sensitization and clonal expansion of C57BL6 splenocytes (effector cells) in response to irrDBA-2 splenocytes (sensitizing cells). By calculating the IC₅₀ for this drug effect, it is possible to compare the relative sensitivities of different subsets of CTL to ZDV and other drugs.

Drug IC₅₀ determination. To define the IC₅₀ of ZDV, data points from the IC₅₀ assay were fit to the logistic model (6) by nonlinear regression using the computer program JUMP-IN (SAS Institute Inc.). The logistic model uses the formula α - β₀/[(1 + β₁ × log dose) + β₂] where α was the fitted log dose, and β₀, β₁, and β₂ were adjustable parameters set initially to equal 1 before the fitting iterations, and log dose was the log10 of the ZDV concentration (in micromolar) used in the assay. A fitted curve was plotted on an x-y axis where α was the x value and fa was the y value (see Fig. 3). fa was defined as the fraction affected by drug.
EFFECT OF ZDV ON NAIVE VERSUS PRIMED CTL

Results

The IC_{50} assay can be used to determine the relative inhibitory activity of antiviral drugs on CTL sensitization. An in vitro system to generate CTL was used to titrate the effects of ZDV on the CTL response. Figure 2 illustrates the results of such an assay. A constant number of effector and stimulator cells in replicates of six were incubated with various concentrations of ZDV during antigen priming. As the ZDV concentration increased, the killing capacity of CTL cultures was reduced in a concentration-dependent sigmoid pattern. From these data, the IC_{50} value for ZDV inhibition of CTL activity was computed by determining the median effective concentration. Figure 3 is an example showing a best-fit plot for determining the IC_{50} value.

Naive CTL are more sensitive to ZDV inhibition than antigen-primed CTL given a second antigen exposure, but less sensitive than antigen-primed CTL held in culture without a second antigen stimulation. In these experiments we compared the inhibitory effects of ZDV on naive versus double-prime or prime/hold effector cell cultures. Results of five independent experiments are shown in Table 1. Individual rows indicate experiments performed at the same time. The IC_{50} values from four independent trials using naive CTL ranged between 79 and 99 μM (mean, 87.5 μM) ZDV (Table 1, Arm 1). By comparison, the IC_{50} values for the four trials with double-prime CTL ranged between 62 and 638 μM (mean, 316 μM) ZDV (Table 1, Arm 2). However, when the antigen-primed C57BL6 CTL were cultured with syngeneic irradiated C57BL6 feeder cells (prime/hold) instead of the double-prime exposure, they were found to be profoundly sensitive to ZDV inhibition, with IC_{50} values ranging from 29 to 30 μM (mean, 29.6 μM) ZDV (Table 1, Arm 3).

ZDV exposure of CTL during primary antigen stimulation does not select for a more ZDV-resistant CTL population. The IC_{50} values of alloantigen-primed effector cells propagated in the presence of 80 μM ZDV were calculated to determine if ZDV treatment during antigen priming selected for a CTL population that was more resistant to the suppressive effects of ZDV. As previously observed, the number of cells collected at the end of the 14-day incubation period in the cultures incubated with ZDV were substantially less than those collected from cultures without ZDV (data not shown). The addition of ZDV to the culture system during the original antigen priming period did not select for a cell population with increased resistance to ZDV suppression. In each of the six paired trials where lymphocytes were primed in the presence of 80 μM ZDV (three in Arm 4 and three in Arm 5), the IC_{50} values were the same or less than the IC_{50} values for the lymphocytes primed in the absence of ZDV. It is clear from these assays that preincubation of T cells during alloantigen priming did not produce a more drug-resistant T-cell population.

In summary, effector cells which were alloantigen primed for 14 days but not restimulated with DBA-2 alloantigen during the IC_{50} assay (incubated with the irradiated C57BL6 feeder cell layer) were most sensitive to ZDV, independent of the concentration of ZDV they encountered during the priming period. Unprimed lymphocytes ranked second in sensitivity,
followed by alloantigen-primed and restimulated effector cells when treated with ZDV during the priming phase. Alloantigen-primed and restimulated effector cells not treated with ZDV during the priming phase were significantly less sensitive to ZDV suppression than unprimed or primed cells that did not reencounter the antigen.

**DISCUSSION**

Recognized limitations of ZDV antiviral therapy are the clinical side effects and the development of drug resistance (4, 37, 51). In addition, both in vitro and in vivo studies have documented that cells of the immune system are sensitive to therapeutic concentrations of ZDV (5, 14, 32, 36, 45, 52). Evaluation of immunomodulation by antiviral drugs on lymphocyte effector function may be crucial because drug-induced interference of immune function, already impaired by virus infection, may potentially reduce even further the capacity to respond to the infection (29, 54).

We previously demonstrated a dose-dependent inhibition of CTL clonal expansion by ZDV in an in vitro alloantigenic murine system (18). In the work described here, using the same alloantigen system, we have introduced a statistical method to determine the IC$_{50}$ of ZDV on antigen-specific cytotoxic T-cell clonal expansion. Based on previous observations by ourselves and others, we hypothesized that the sensitivity of T lymphocytes to the suppressive effects of ZDV depends on the activation status of those cells (naive versus antigen primed).

Our results revealed that 80 to 100 µM ZDV reduced the capacity of naive splenocytes to become CTL effector cells by one-half (mean IC$_{50}$ = 87.5 µM). By contrast, antigen-primed lymphocytes with continuous exposure to allogeneic cells (double prime) were substantially more resistant to the suppressive effects of ZDV (mean IC$_{50}$ 316 µM). Antigen-primed CTLs differ from their naive precursors in many regards, including morphology, surface antigen expression, and responsiveness to antigen reexposure and cytokine expression, e.g., IL-2 (33, 39).

It is known that antigen-activated murine CTL do not require additional contact with stimulatory antigen for continued clonal expansion but respond to mitogenic lymphokines for a defined time period after antigen contact (1). Therefore, substituting syngeneic irradiated C57BL6 splenocytes as feeder cells for alloantigenic DBA-2 splenocytes during the IC$_{50}$ assay following antigen priming permitted evaluation of cytolytic precursors which became activated during the primary antigen encounter (first 14 days of culture). By contrast, cultures which were primed during the first 14 days and were reexposed to alloantigen during the inhibition assay allowed detection of both primed effector cells and antigen-specific precursors which were not stimulated during the first antigen exposure (43). With this in mind, we found that alloantigen-primed lymphocytes, reexposed to the same antigen (double prime), were less sensitive to the suppressive effects of ZDV (higher IC$_{50}$) than primed cells not reexposed to antigen (prime/hold).

Since the maintenance of antigen-primed but nonrestimulated effector cells is mainly dependent on IL-2 utilization (28, 44), ZDV interference with either receptor expression or receptor-ligand interaction would be potential mechanisms explaining the observed difference in sensitivity. Exogenous IL-2 reverses or reduces the in vitro and in vivo suppressive effects of ZDV, consistent with this hypothesis (36, 40, 45, 47). However, a separate study, which specifically evaluated cytokine production by lymphocytes from HIV-positive patients during ZDV treatment, did not reveal reduced IL-2 production (31), and another study reported a significant increase in IL-2 receptor (CD25) expression by mitogen-stimulated T lymphocytes from ZDV-treated AIDS patients (38). Therefore, even though excess exogenous IL-2 was provided in the tissue culture medium of our cultures during the antigen priming period and in the IC$_{50}$ assay, we cannot exclude the possibility that ZDV interfered with cytokine-dependent T-cell-mediated helper function.

We further found that 80 µM ZDV treatment of CTL during antigen priming had a drug concentration-related effect on the number of cells harvested after the 14-day priming period (data not shown) but did not affect the ZDV IC$_{50}$ values of progeny cells compared to non-ZDV-treated cells. This observation was independent of the type of antigen (syngeneic or allogeneic) used during the culture period. We had previously shown that the concentration-response-related reduction of effector cells harvested following priming in the presence of ZDV was most likely mediated by reduced clonal expansion of alloantigen-stimulated effector cells (18). However, based on the present study, it appears that previous antigen exposure rendered the effector cell more sensitive to the suppressive effects of ZDV (prime/hold study). This sensitivity was altered

**TABLE 1. Effect of alloantigen priming on the IC$_{50}$ of ZDV-mediated reduction of CTL function**

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Arm 1</th>
<th>Arm 2</th>
<th>Arm 3</th>
<th>Arm 4</th>
<th>Arm 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naive</td>
<td>No ZDV during first antigen exposure</td>
<td>Prime/hold</td>
<td>Double prime</td>
<td>Prime/hold</td>
</tr>
<tr>
<td>1</td>
<td>79.3 (48–91)</td>
<td>638 (481–880)</td>
<td>ND</td>
<td>324 (267–387)</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>91 (61–134)</td>
<td>353 (304–389)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>99 (81–115)</td>
<td>211 (155–225)</td>
<td>29 (12–43)</td>
<td>29 (21–37)</td>
<td>31 (26–35)</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>62 (46–74)</td>
<td>30 (26–32)</td>
<td>70 (66–78)</td>
<td>33 (28–37)</td>
</tr>
<tr>
<td>5</td>
<td>81 (47–115)</td>
<td>ND</td>
<td>30 (23–36)</td>
<td>ND</td>
<td>24 (18–30)</td>
</tr>
<tr>
<td>Mean</td>
<td>87.5</td>
<td>316</td>
<td>29.6</td>
<td>141</td>
<td>29.3</td>
</tr>
</tbody>
</table>

a Shown are results from five independent trials. Individual rows indicate experiments performed at the same time.
The mechanism by which ZDV suppresses CTL leading to variable IC_{50} values for naive, prime/hold, and double-prime CTL is not known. Based on previous studies, it is assumed that ZDV suppresses clonal expansion of antigen-stimulated CTL (18). ZDV has been shown to prolong mitosis (10, 49), inhibit mitochondrial DNA polymerase (9, 13, 35, 41), and deplete the TTP pool (19), all of which affect cell proliferation. In our previous studies (18), we found ZDV to be cytostatic at concentrations ranging between 15 and 250 \mu M and cytotoxic at concentrations of 500 \mu M or greater when tested on naive lymphocyte cultures. These results were gathered using the same C57BL/6/DBA-2 alloantigen model (18). We also observed that a delay in the addition of ZDV reduced its suppressive effect in vitro (18) and in vivo (34), allowing more effector cells to be produced.

Tissue culture cell lines as well as peripheral blood mononuclear cells (PBMC) grown long-term in the presence of ZDV become resistant to ZDV antiviral activity by decreasing the concentration of thymidine kinase (TK), a crucial enzyme necessary for the phosphorylation of ZDV to ZDV-monophosphate (ZDV-MP). The failure of the phosphorylation cascade necessary to convert ZDV to its active anabolite, ZDV-triphosphate (ZDV-TP), renders the cells less sensitive to the antiviral activity of ZDV and less sensitive to the toxic effects of ZDV caused by the ZDV-MP intermediate (20, 48). Reduced TK expression in ZDV-resistant Jurkat T cells has been linked to methylation of the human TK gene (52). The observation that PBMC from HIV-infected patients on long-term ZDV have reduced TK is suggestive that ZDV modulates TK expression (22). However, a more likely explanation is that ZDV therapy selects for a T-cell population of naturally low expressors of TK. Phytohemagglutinin-stimulated human PBMC have higher TK expression and greater sensitivity to ZDV inhibition of HIV-1 infection than nonstimulated controls (21). These cells are likely to be more sensitive to the cytostatic and cytotoxic effects of ZDV (low IC_{50}). This latter observation is counter to our work, where highly activated T cells (double prime) had greater resistance to ZDV toxicity.

An alternative explanation for increased resistance to ZDV by activated T cells is increased expression of the multidrug-resistant (MDR) transmembrane P-glycoprotein (p170), which acts as an efflux pump to remove intracellular ions, toxins, and drugs (15), rendering the cell resistant to the cytostatic and cytotoxic effects of ZDV (2, 3, 12, 53). In normal human PBMC, the majority of CD8^{+} T cells, but less than half of the CD4^{+} T-cell population, express p170 (11). Interestingly, phytohemagglutinin stimulation of human PBMC causes an increase in p170 by the CD8^{+} T-cell subset, and anti-p170 blocks the cytolytic activity of alloantigen-specific cytotoxic T cells (23). A natural function of p170 may be in aiding the secretion of certain cytokines (24). Taken together, it appears that p170 expression is easily modulated by immune stimulation and may play an important role in cytotoxic T-cell effector function. The upregulation of p170 by antigen-stimulated T cells, as a mechanism for increased drug resistance, would be compatible with our double-prime stimulation results. Further studies will be needed to document the full range of p170 expression in the different activation stages of cytotoxic T cells and to correlate those results with drug sensitivity.

Determining the relevance of this work to the plasma ZDV concentrations of humans on ZDV therapy is made difficult by the species differences in ZDV processing. Mouse lymphoid cells form ZDV-TP at a level 16 times higher than that of human lymphoid cells (6). Human lymphoid cells were reportedly 15 times more sensitive to the cytostatic effects of ZDV and dideoxycytosine than were mouse lymphoid cells (6). The high level of ZDV-MP formed in human lymphoid cells, which is known to inhibit thymidylate kinase and adenylate kinase (7), may account for the cytostatic effect of ZDV. Therefore, studies in mouse systems may underestimate the true cytostatic effect of ZDV treatment in human cells by a factor of 15. The concentration of ZDV found to suppress the CTL response of mouse splenocytes by 50% ranged between approximately 30 and 600 \mu M (Table 1), while the peak ZDV concentration in patients receiving the recommended ZDV dosage has been calculated to be in the range of 3 to 7 \mu M. Assuming the 15-fold difference between mice and human cells, one might predict that human naive T cells would be suppressed by ZDV.
concentrations as low as 2 μM, well within the human peak plasma drug concentration. These assumptions, however, are based on predicted behavior of human lymphocytes and not actual T-cell response data. The IC50 assay used in a mouse alloantigen model will be a useful tool for testing the drug sensitivity of human T-cell responses to HIV antigens.

Taken together, our studies show that ZDV reduced cytolytic effector cell function in a concentration-dependent manner and suggest that alloantigen-primed effector cells, when not given continuous exposure or rescued by a second antigen encounter, are more sensitive to ZDV suppression than naive cells. This observation may have relevance in persons with HIV, where a strong CTL immune response is critical for the prevention of disease progression (25, 30, 46). Administration of ZDV and possibly other nucleoside analogues during the time of initial antigen priming of T cells may reduce the peak CTL response to virus. Possible drug interference with immune function should be considered when determining drug dosage and the time point of ZDV treatment initiation.

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