Effect of Zidovudine on the Primary Cytolytic T-Lymphocyte Response and T-Cell Effector Function

SABINE FRANCKE,† CHARLES G. OROSZ,2,3,4 KATHLEEN A. HAYES,1,3 AND LAWRENCE E. MATHES1,3,4,5

Department of Veterinary Biosciences, College of Veterinary Medicine,1 Department of Surgery, College of Medicine,2 Center for Retrovirus Research,3 and Comprehensive Cancer Center,4 The Ohio State University, Columbus, Ohio 43210

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Azidothymidine (AZT) and other nucleoside analogues, used to treat AIDS, can cause severe clinical side effects and are suspected of suppressing immune cell proliferation and effector immune cell function. The purpose of the present study was to quantitatively measure the effects of AZT on cytotoxic T-lymphocyte (CTL) priming and to determine if the major histocompatibility complex-restricted CTL killing was affected by AZT exposure. For this purpose, we employed a murine alloantigen model and limiting-dilution analysis (LDA) to estimate cytotoxic effector cell frequencies of alloreactive splenocytes treated with drug during antigen sensitization. This noninfected model was chosen to avoid analysis of a virus-compromised immune system. Exposure of splenocytes to therapeutic concentrations of AZT (2 to 10 μM) caused a two- to threefold dose-dependent reduction in CTL precursor frequency. This reduction was caused by decreased proliferation of alloantigen-specific CTLs rather than loss of function, because full cytotoxic function could be restored by adjusting the AZT-treated effector/target cell ratios to that of untreated cells. In addition, when AZT was added to the assay system at various times during antigen sensitization there was a time-related loss of the suppressive effect on the generation of cytolytic effector function, suggesting that functional CTLs are not affected by even high doses of AZT. Taken together, the data indicate that the reduction of CTL function associated with AZT treatment is due to a quantitative decrease of effector cell precursor frequency rather than to direct drug cytotoxicity or interference with mediation of cytolyis. Furthermore, antigen-naive immune cells were most sensitive to this effect during the first few days following antigen encounter.

Zidovudine (azidothymidine [AZT]) has profound antiviral activity against human immunodeficiency virus (HIV) and other retroviruses (18) and continues to be an important therapeutic for treating HIV-positive pregnant women and their postpartum infants (4, 8). AZT, however, is known to suppress lymphocyte responses to viral and nonviral antigens, mitogens, and anti-CD3 antibody stimulation (11, 14, 28). In previous studies, we and others have protected feline leukemia virus (FeLV)-infected cats from chronic infection by pre- and post-exposure prophylactic AZT therapy (16, 31). The protective effect was thought to be a “drug-induced vaccine effect,” where AZT treatment during primary infection suppressed virus expression and permitted protective immunity to develop (16). Similar effects have been described for a murine retrovirus model (25, 26). Interestingly, when cats were given the same AZT prophylactic regimen preceded by a loading dose of AZT close to the time of FeLV challenge, the protective effect was lost (15). We hypothesized that high-dose AZT at the time of infection suppressed the primary immune response, permitting rapid virus expansion and a loss of immune protection. The interpretation of these results, however, was complicated by the simultaneous FeLV infection, which was immunosuppressive independent of drug therapy (7, 23). Similar problems are associated with assessing drug-related immunootoxicity in AIDS patients as well as other virus challenge systems which may modulate immune responses. The possible immunomodulatory effects of antiviral therapy in AIDS patients is important because strong cytolytic lymphocyte responses in HIV-infected individuals is associated with long-term survival (24). Currently, the molecular basis for the suppressive effect of AZT on immune cells is poorly understood, and the degree to which major histocompatibility complex (MHC)-restricted cytotoxic T-lymphocyte (CTL) responses are affected is unknown.

The current study was undertaken to determine if therapeutic concentrations of AZT suppress antigen-specific MHC-restricted CTL proliferation and effector CTL function in a non-infectious system.

**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 eight-week-old female DBA/2 (H-2 d Mlsa) and C57BL/6 (B6; H-2 b Mlsb) mice were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, Ind.). The mice were housed in a laminar flow cabinet (Animal Storage Isolators; Nu Aire, Inc., Plymouth, Minn.) in groups of 5 to 10 animals per cage. AZT, AZT was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, as a lyophilized powder and stored in light-protected conditions at room temperature. AZT was dissolved in Dulbecco modified Eagle medium supplemented with 1.6 mM L-glutamine, 0.27 mM L-arginine, 0.27 mM L-asparagine, 0.55 mM L-lysine, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, and 10 U of interleukin-2 (IL-2) (Roche Molecular Biochemicals, Indianapolis, IN). Cells were grown in Dulbecco modified Eagle medium supplemented with 1.6 mM L-glutamine, 0.27 mM L-arginine, 0.27 mM L-asparagine, 0.55 mM L-lysine, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, and 10 U of interleukin-2 (IL-2) (Roche Molecular Biochemicals, Indianapolis, IN).
microcultures were considered positive for cytolysis when the mean $^{51}$Cr release that failed to produce detectable cytolytic activity (10, 19, 29). Limiting-dilution to the limiting-dilution microwells and the percentage of replicate microwells Poisson distribution relationship between the number of responder cells added inactivated fetal bovine serum, 100 IU of penicillin/ml, and 100 parts of Leibovitz L-15 and RPMI 1640 and supplemented with 10% heat-cytotoxic activity of the two cell populations. P815 cells were propagated in equal volumes of cell suspension was transferred from each well, without adjustment of 3 for measuring MHC-restricted CTL responses but will not serve as target cells for the NK cell cytotoxicity (21), thus permitting the distinction between the cytotoxic activity of the two cell populations. P815 cells were propagated in equal parts of Leibovitz L-15 and RPMI 1640 and supplemented with 10% heat-inactivated fetal bovine serum, 100 IU of penicillin/ml, and 100 µg of streptomycin/ml and then incubated at 5% CO2 and 37°C. For $^{51}$Cr labeling, a cell pellet of 3 $\times$ 10$^6$ P815 cells was resuspended in 650 µCi of $^{51}$CrCl2 (New England Nuclear, Boston, Mass.) in a total volume of approximately 100 µl, incubated at 37°C in 5% CO2 for 2 h, washed up to eight times in PBS, and adjusted to the required cell concentration.

In vitro sensitization and cytolytic activity. Three related protocols (limiting-dilution analysis [LDA] and nonadjusted and adjusted CTL microculture assay), represented in Fig. 1, were used to measure cytolytic activity of in vitro-sensitized lymphocytes. In all experiments, lymphocytes were pooled from three to eight mice. All assays were performed in multiple replicates, and each assay was carried out in at least three separate studies.

LDA. The limiting-dilution microculture conditions for estimating the frequency of CTL effector cells were adapted from methods previously described (22). Serial twofold dilutions of responder C57BL/6 splenocytes, beginning at 3 $\times$ 10$^3$ cells/well and continuing for eight dilutions, were added to 96-well, V-bottom microculture plates to test cytotoxic activity by $^{51}$Cr-release assay. A total of 3 $\times$ 10$^3$ $^{51}$Cr-labeled P815 target cells in a 100-µl volume was added to each well. Cultures were incubated for 7 days at 37°C in 10% CO2. On the seventh day, 100 µl of cell suspension was transferred from each well, without adjustment of effector cell numbers, to V-bottom microculture plates to test cytolytic activity by $^{51}$Cr-release assay. A total of 3 $\times$ 10$^3$ $^{51}$Cr-labeled P815 target cells in a 100-µl volume was added to each V-bottom well. After a 5-h incubation period at 37°C in 10% CO2 and centrifugation, 100 µl of cell-free supernatant was harvested from each microculture well and measured for $^{51}$Cr release using a gamma counter (Beckman Instruments, Fullerton, Calif.). As previously reported (22), minimal estimates of the CTL frequency were calculated by analysis of the Poisson distribution relationship between the number of responder cells added to the limiting-dilution microcultures and the percentage of replicates microwells that failed to produce detectable cytolytic activity (10, 19, 29). Limiting-dilution microcultures were considered positive for cytolysis when the mean $^{51}$Cr release for the CTL assay was greater than the mean plus three standard deviations (SDs) of the $^{51}$Cr release for the DBA/2 plus IL-2 control (spontaneous release). Frequency calculations were made using the zero-order term Poisson equation linearized to the form $Y = F - x \ln A$ (where $x$ is the number of responder cells/well, $Y$ is the percentage of negative microwells, $F$ is the CTL frequency defined as the negative slope of the line, and $A$ is the y-axis intercept). Experimental $x$ and $y$ values were computer fitted to the equation by chi-square minimization analysis (29) using a program written for this purpose (C. Oroz). This analysis provides a frequency estimate ($f$), a frequency range for 95% confidence limits (range), and a chi-square analysis of the frequency estimate probability ($P$). It is important to note that in this system a statistically acceptable frequency estimate has a $P$ value of $\leq 0.05$.

Nonadjusted CTL microculture assay. Nonadjusted CTL assays used a culturing protocol similar to that used in the LDA assay where effector cell numbers were not adjusted after the 7-day antigen-priming period. However, cultures were in replicates of 6 instead of 24 wells, and only one effector cell concentration, corresponding to the highest cell concentration used in the LDA, was evaluated. A total of 3 $\times$ 10$^3$ C57BL/6 splenocytes in 100 µl and 10$^3$ irradiated DBA/2 spleen cells in 100 µl was added to each well of U-bottom microculture plates and allowed to incubate for 7 days. During the 7-day coculture period, AZT at concentrations of 0, 10, 50, 100, 200, and 500 µM was added to separate culture plates on day 1, 3, 4, 5, 6, and 7. After 7 days, the cultures were harvested as described above and evaluated for cytotoxic activity.

Adjusted CTL microculture assay. In the adjusted CTL assay, equal numbers and volumes (10$^3$ cells/100 µl) of C57BL/6 and irradiated DBA/2 spleenocytes were added to U-bottom microculture plates and incubated for 7 days at 37°C in 10% CO2. The U-bottom plate culture format was used to simulate the culturing conditions found in the LDA assays. Cells were then harvested, pooled, and counted. Effector cells from these cultures were adjusted to achieve a final effector/target cell (E:T) ratio of 100, 80, 40, 20, 10, 5, 1, and 0.5 and distributed into V-bottom 96-well plates in a volume of 100 µl. Each cell concentration had six replicates. A total of 3 $\times$ 10$^3$ $^{51}$Cr-labeled P815 target cells in a 100-µl volume was added to each V-bottom well. The cultures were incubated for 5 h as described above and assessed for $^{51}$Cr release.

Determination of cell viability and total cellularity. Cell viability was assessed using the Trypan blue dye exclusion technique. A total of 3 $\times$ 10$^5$ C57BL/6 cells and 10$^3$ irradiated DBA/2 cells each in 100 µl were cocultured in U-bottom microculture plates in the presence of AZT at concentrations ranging from 0 to 1 µg/ml for up to 7 days as described above. Control wells contained stimulator cells plus IL-2 only. Separate plates were counted on each of the 7 days of culture.

### Table 1. LDA of AZT-treated CTL precursor frequency

<table>
<thead>
<tr>
<th>Expnt. no.</th>
<th>AZT (µM)</th>
<th>CTL frequency (range$^{-1}$)</th>
<th>Frequency estimate (range$^{-1}$)</th>
<th>No. of CTL/M$^{10^5}$ PBMC</th>
<th>P</th>
<th>% Control response</th>
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<tr>
<td>1</td>
<td>0</td>
<td>502</td>
<td>385–721</td>
<td>1,992</td>
<td>0.519</td>
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<td>2</td>
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<td>5</td>
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<td>758</td>
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<tr>
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<tr>
<td>2</td>
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<td>603–1,189</td>
<td>1,248</td>
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<td>3</td>
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<td>&gt;300,000</td>
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RESULTS

AZT induces a dose-dependent reduction of the estimates of the CTL precursor frequency of alloantigen-stimulated lymphocytes. LDA was used to determine the effect of AZT on the predicted frequency of alloantigen-specific CTL precursors. Because the LDA depends on precursor cell proliferation, the assay is useful for measuring drug effects on the primary immune response. Table 1 shows the results from three independent trials in which various concentrations of AZT were added to the antigen priming phase of the in vitro LDA. Each trial showed a dose-dependent reduction of the estimate of the alloantigen-reactive CTL precursor frequency. The data from one of these experiments (experiment 2) is shown in Fig. 2. Figure 2A is the plot of a typical LDA assay showing the drop in the number of wells with positive cytolysis as the number of effector cells per well is reduced. Wells with a $^{51}$Cr activity of >3 SD above the spontaneous release (the lower horizontal dashed lines in Fig. 2A and B) were considered positive. Figure 2B is an identical assay except that the lymphocytes were sensitized in medium containing 100 μM AZT. The estimate of CTL precursor frequency was reduced by approximately 10-fold in the presence of 100 μM AZT. Figure 2C is a precursor frequency graph displaying the comparative data collected from Fig. 2A and B. Overall, the effects on CTL precursor frequency were modest (two- to threefold reduction) for doses of <100 μM AZT (Table 1).

AZT reduces alloantigen-stimulated cell proliferation in a dose-dependent fashion. The reduced frequency of alloantigen-specific CTL precursors could be due to mechanisms such as AZT cytotoxicity against naive precursor cells, AZT suppression of T-cell proliferation in response to alloantigen, and/or AZT interference with mediation of cytolysis. To determine if AZT-treated CTL cultures had reduced proliferation, viable cell counts were taken daily from C57B/6-DBA cocultures during the 7-day in vitro sensitization period with or without various concentrations of AZT. The microcultures showed a dose-dependent effect on cell viability throughout the 7-day incubation period (Fig. 3). Initially, viable cell counts in all cultures dropped due to the combined deaths of both the irradiated stimulator cells (DBA) and the nonresponder lymphocytes. By days 4 to 5, antigen-specific lymphocytes began to proliferate, with a concomitant rise in total viable cell counts. AZT suppressed the proliferation of effector cells in a dose-dependent manner over days 5, 6, and 7. Separate cell counts of the number of dead cells in these cultures demonstrated that AZT concentrations of 500 and 1,000 μM were lethal (data not shown). A second independent assay produced similar results. Thus, AZT-treated cultures had fewer proliferating lymphocytes than did the controls.

AZT does not affect cytolytic effector function. Although AZT reduced CTL precursor proliferation in a dose-dependent fashion, it was important to determine if CTL function was likewise affected. To address this issue, CTL microcultures, sensitized in the presence or absence of AZT, were adjusted to specific E:T ratios (adjusted CTL microculture assay [Fig. 1]) after sensitization but prior to assessment of cytolysis. CTLs generated in the presence of various AZT concentrations (0 to 100 μM) were harvested and washed to remove AZT. Cell concentrations were then adjusted to predetermined effector cell numbers based on viable cells only. As shown in Fig. 4, adjusting cell counts to the same numbers of effector cells resulted in approximately equal capacity to mediate maximal cytolysis irrespective of the amount of AZT present during the antigen sensitization phase. As the E:T ratio was lowered to 10:1, the cytolytic activity declined accordingly. These results support the contention that the reduction in effector cell frequency was due to a reduction in the proliferation of effector cells rather than direct interference with effector cell function. The results also suggest that AZT at concentrations of up to 100 μM was cytostatic rather than lethal to effector cells because viable and functional effector cells were still present but in lower numbers in cultures treated with higher doses of AZT.

The immunomodulatory effect of AZT is most pronounced during the initial antigen sensitization period of CTL precursor generation. Additional studies were carried out to determine if delaying the time of drug addition with respect to antigen priming would affect the generation of CTL. For this purpose doses of AZT ranging from 0 to 500 μM were added to nonadjusted CTL microculture assays (Fig. 1) on various
days of culture (day 1, 2, 3, 4, 5, 6, and 7). On day 7, the cultures were tested for their cytolytic response patterns by $^{51}$Cr release. AZT concentrations of 10 and 50 µM did not influence the generation of cytolytic function unless added at day 1 of culture compared to the control containing no AZT (Fig. 5). Concentrations of 100, 200, and 500 µM had a time-dependent effect on CTL generation early, but starting at day 5 there was no apparent effect at any of the drug concentrations tested.

**DISCUSSION**

It is now widely believed that the HIV-specific CTL response plays an important role in combating early virus spread and contributes to long-term survival (24). Therefore, it is important to define the effects of antiviral drugs such as AZT on immune cell function to avoid possible drug-mediated impediment of the immune system. Our approach has been to evaluate antiviral agents for immunomodulation in a system not compromised by virus infection.

The goal of the present study was to define the effect of AZT on MHC-restricted CTL functions by using limiting dilution analysis in an allogeneic mouse model. This model is well established, reproducible, and very sensitive based on an intrinsically high precursor frequency of alloreactive cells compared to responses to other antigenic stimuli (12, 19, 22). The limiting dilution technique provides a minimal frequency estimate of inducible CTL precursors present in a given lymphocyte population (12, 29) and represents a naive immune response at the time of primary antigen exposure. This system also permits distinction between MHC-restricted CTL and NK cell killing because P815 cells, the target cell line for the CTL assay, are not subject to NK cell killing (21).

Our investigation demonstrated that AZT reduced the predicted frequency of MHC-restricted CTL in alloantigen-stimulated cultures by blocking clonal expansion of alloantigen specific CTL. In LDA assays, a range of 2 to 400 µM AZT caused a 2- to >20-fold reduction in the number of CTL.
precursors responding to antigen in a dose-dependent manner (Table 1). Previous studies in mice and humans have documented the suppressive effects of AZT on immune cell killing at relatively high doses and using mixed-lymphocyte-culture (MLC)-bulk culture conditions (11, 27, 30). These studies, however, failed to define the stage of T-cell development sensitive to AZT-mediated suppression. The present report shows impaired proliferative capacity of the AZT-treated cells as the most likely cause for a reduced frequency of CTL precursors and support the observation that AZT is cytostatic and not cytotoxic or lethal at concentrations of up to 200 μM (5). Our own viability studies discount lymphocyte death as the cause of reduced CTL killing for AZT concentrations of ≤125 μM.

An additional experiment was performed to rule out interference with maturation and differentiation of effector cells as the cause of reduced CTL activity. For that purpose, cells preexposed for 7 days to different concentrations of AZT during antigen sensitization were washed, and the number of viable cells was adjusted to defined E:T ratios on the day of the assay for cytolyis (Fig. 4). By adjusting the effector cell concentration to equivalence for the various AZT-treated cultures, we compensated for any reduction in effector cell numbers due to AZT suppression of proliferation. These cultures showed no loss in effector cell function for the AZT-treated cultures.

We further investigated the possibility that lymphocytes had a differential sensitivity to the effects of AZT depending on their degree of maturation at the time of drug encounter (Fig. 5). When AZT was added to C57BL/6-DBA priming cultures on consecutive days following primary antigen encounter, cells were most sensitive to the suppressive effects of AZT closest to the time of antigen priming. This suppressive effect was lost when AZT was added to the culture system later than day 4 of our experiment. We interpret these results to indicate that the priming step occurring in the first 24 to 48 h of culture is the stage most sensitive to the AZT effect. An alternative explanation is that the longer exposure to AZT is more effective in suppressing proliferation and CTL activity. The observation that the drug effect was dose dependent tends to support the former rather than the latter explanation. Similar studies were reported by McKallip et al. in a comparable mouse alloantigen system (17).

Taken together, the data point to an undifferentiated, naïve cell population as the primary target of the suppressive effects of AZT and to inhibition of clonal expansion as the most likely mechanism by which CTL precursor cell frequency is reduced. Others (17, 27) who made similar observations in MLC systems, reasoned that since clonal expansion is known to be mainly a response to cytokines, interference with IL-2 production by T cells after antigen encounter could be the underlying mechanism for the phenomenon observed. This argument was supported by the demonstration that adding exogenous IL-2 reduced (27) or completely eliminated (17) AZT-mediated effects on cytolytic immune cell function. However, in our study, using LDA microcultures, IL-2 was added in excess, suggesting that the suppressive effect observed was not IL-2 dependent. In contrast, cytokines are produced de novo by the various cell subsets present in the conventional MLC assay. Nevertheless, it remains possible that AZT has inhibitory effects on helper T cell functions leading to an imbalance of critical cytokine patterns needed for proper clonal expansion of CTLs in response to antigenic stimulation.

The efficacy of nucleoside analogue therapy is host and cell type dependent. Nucleoside analogues are phosphorylated by host cell origin phosphokinases to their mono-, di-, and triphosphate metabolites. Cell lines derived from different species as well as different cell types from the same species metabolize nucleoside analogues differently. In previous studies with AZT and ddI, we demonstrated that U937 cells (human monocytic) were more efficient in AZT triphosphate (AZT-TP) synthesis than Molt-4 cells (human lymphoid) (20). Others have shown similar differences between mouse and human lymphoid cells where mouse cells formed AZT-TP at a level 16 times higher than human cells (1). However, in spite of the low concentrations of AZT-TP in human cells, it is noteworthy that human lymphoid cells were 15 times more sensitive to the cytostatic effects of AZT and ddC than were mouse lymphoid cells (1). The high level of AZT monophosphate (AZT-MP) formed in human lymphoid cells, which is known to inhibit thymidylate kinase (32) and adenylyl kinase (2), may account for the cytostatic effect of AZT. Thus, studies in the mouse system may underestimate the true cytostatic effect of AZT treatment on human cells by a factor of 15. Our studies with mouse cells used AZT concentrations of between 2 and 400 μM, while the peak AZT concentrations achieved in patients receiving the recommended AZT dosage is calculated to be in the range of 3 to 7 μM (6). Within this range we observed statistically significant but modest reductions (two- to three-fold) in CTL precursor frequencies for mouse lymphocytes. Whether human T-lymphocyte precursors show the same degree of sensitivity to AZT has not been adequately documented. Viora et al. reported the suppression of ³H-labeled thymidine uptake in human peripheral blood mononuclear cells (PBMC) and CEM cells by AZT in a dose-dependent manner (33). However, AIDS patients responding to AZT therapy showed a rapid increase (up to a 100-fold) in their HIV-specific CTL response (9). Whether this increase in CTL activity was related to expansion of an HIV-sensitized memory cell population, stimulation of naïve T cells, or redistribution of sequestered HIV-specific CTLs (3) is not known. In a more
AZT Suppresses CTL Responses in Vitro

Recent study, patients receiving highly active antiretroviral therapy showed a decline in the level of in vivo-activated HIV-1-specific CTLs, presumably as a result of almost complete elimination of plasma virus and therefore antigen stimulation (13). It should also be noted that each of the nucleoside analogues found to have antiviral activity may show strikingly different efficacies when tested in different species or even different cells from the same species (1, 20, 32). Therefore, the immunomodulatory activity of each agent should be considered separately.

In summary, we have shown a direct correlation between the qualitative and quantitative immunomodulatory effects of AZT on CTL effector functions in vitro. AZT did not appear to interfere with maturation and activation of CTL precursors but did suppress clonal expansion following antigen priming.

Acknowledgments

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