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3′-AZIDO-3′-DEOXYTHYMIDINE INHIBITION OF HUMAN LYMPHOCYTE CYTOLYTIC FUNCTION *IN VITRO*

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Abstract—Despite administration of 3′-azido-3′-deoxythymidine (AZT, Zidovudine) to seriously immunocompromised patients, little has been reported regarding effects of AZT on specific immune functions. This study analyzed the *in vitro* effect of AZT on normal human lymphocyte cytolytic activity. AZT at concentrations up to 100 μM had no effect when added directly to cytotoxicity assays with lymphocyte effector cells and natural killer (NK)-sensitive or NK-resistant target cells. In contrast, addition of AZT to lymphocytes cultured for 4–10 days with interleukin-2 (IL-2) prior to cytotoxicity assays produced a concentration- and time-dependent inhibition; this effect was not mimicked by acyclovir or ganciclovir. Lymphocyte cell numbers and viability were not reduced in parallel to inhibition of cytolytic activity by AZT. Furthermore, AZT inhibition of IL-2-dependent cytolytic activity was not correlated with alterations in lymphocyte cell surface phenotypes by flow cytometry, and lymphocyte culture supernatant levels of interferon-γ were not reduced by AZT. These results suggest that AZT may selectively inhibit human lymphocyte functions and thus may have implications for long-term therapeutic administration of AZT in chronic immunodeficiency states.

3′-Azido-3′-deoxythymidine (AZT or Zidovudine) inhibits retrovirus proliferation *in vitro* and has demonstrated clinical benefit in the management of syndromes (AIDS and ARC) associated with human immunodeficiency virus (HIV) infection [1, 2]. The active form of AZT is probably the cellular anabolite AZT-triphosphate which inhibits viral reverse transcriptase 100-fold more than mammalian α-DNA polymerase and thus selectively interrupts viral replication [3]. In clinical trials, AZT has been noted to normalize some patients' circulating CD4+ lymphocyte and total lymphocyte counts as well as CD4+/CD8+ ratios during the first 8–12 months of therapy; however, continued AZT therapy may not sustain this presumed beneficial response [1, 2]. Whether "relapse" to abnormal circulating lymphocyte profiles is due to a toxic effect of AZT on lymphocytes or to emergence of AZT-resistant HIV *in vivo* [4] or to other mechanisms is not clear. More importantly, AZT has produced dose-limiting bone marrow toxicities in clinical trials [2] and *in vitro* AZT has been demonstrated to inhibit human hematopoietic progenitor cell proliferation [5–7]. In addition, AZT has been reported to inhibit human lymphocyte mitogen and antigen-specific responses *in vitro* [8–10], indicating that several normal host cell functions may be affected adversely by concentrations of AZT that inhibit viral replication.

In this study, potential effects of AZT on specific human lymphocyte functions were examined. The results indicate that AZT, at a concentration of 10 μM that approximates peak serum levels in clinical trials [1], can inhibit normal human peripheral blood lymphocyte interleukin-2 (IL-2)-dependent cytolytic activity against both natural killer (NK)-sensitive and NK-resistant target cell lines; higher concentrations of AZT, although perhaps not pharmacologically relevant, produced a more profound inhibition. Inhibition was not correlated with generalized lymphocyte toxicity as measured by total and viable cells in lymphocyte AZT cultures, by lymphocyte cell surface antigen phenotypes or by lymphocyte culture production of interferon-γ. Thus AZT, at concentrations below generally cytopathic levels, may exert subtle and perhaps selective deleterious effects on normal human immume functions.

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MATERIALS AND METHODS

Lymphocyte effector cell cultures. Human blood was collected from normal healthy volunteers, mononuclear cells were isolated by Ficoll–Hypaque separation, and lymphocytes were enriched by monocyte adherence as previously described [11]. Lymphocytes were routinely cultured at 10^6 cells/mL in McCoy's 5A medium + 10% fetal calf serum (FCS) and 100 μg/mL gentamicin. Recombinant human IL-2 (Biogen, Cambridge, MA) was added for final concentrations of 1–100 units/mL. AZT (gift of Dr. Raymond F. Schinazi, Emory University, GA), acyclovir and ganciclovir (both provided by Dr. William C. Buhles, Syntex Research) were all added at final concentrations of 1–100 μM. Cultures maintained for 9–10 days were fed twice at 3-day intervals by adding half-volumes of fresh medium containing the same concentrations of IL-2 and/or

drugs. At the end of culture, harvested cells were washed thoroughly, counted and assayed for Trypan blue dye exclusion by microscopic enumeration in a hemacytometer chamber.

Lymphocyte cytotoxicity assays. Indicator target cells for cytolytic assays were the K562 human erythroleukemic line (NK-sensitive), and the Daudi and Raji human B-lymphoblastoid lines (NK-resistant). Target cells were labeled with ^{111}In-indium oxine (1 mCi/mL, >50 mCi/μg indium, Amersham, Arlington Heights, IL) by 10-min incubation at room temperature with 40 μCi/10^7 cells. Targets were washed twice with RPMI 1640 medium + 10% FCS prior to assay. Final labeling of targets was 5000–80,000 cpm/10^4 cells.

Cytotoxicity assays were set up with 1×10^4 ^{111}In-labeled targets in 96-well round-bottom plates. Cultured lymphocyte effector cells (E) suspended in RPMI + 10% FCS were added to target cells (T) for the indicated $E:T$ ratios. Parallel samples contained targets without added effectors to determine non-specific or "spontaneous" release of ^{111}In; spontaneous release was always <20% of total target label in reported experiments. Assays were incubated for 4 hr, supernatants harvested for gamma counting of released ^{111}In (cpm), and results expressed as target cell percent lysis by previously described methods [11] using the following formula:

$$\text{Percent lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100.$$

Lymphocyte surface phenotypes. In parallel with cytotoxicity assays, aliquots of cultured lymphocytes were analyzed for surface antigen expression using specific monoclonal antibodies and indirect immunofluorescence analysis on a Becton–Dickinson FACStar instrument. Analyses were performed with antibodies (Becton–Dickinson) specific for the following antigens: Leu-4 (CD3), Leu-3 (CD4), Leu-2 (CD8), Leu-17 (CD38), Leu-19 (CD56), HLA-DR, and IL-2 receptor (CD25, p55 Tac).

Interferon-γ assay. Cell-free supernatants from the above lymphocyte effector cell cultures were assayed using the Centocor, Inc., γ-interferon radioimmunoassay kit according to the manufacturer's instructions.

RESULTS

We initially examined the effects of AZT added to cultures of normal peripheral blood lymphocytes, with or without exogenous IL-2, on subsequent assays of lymphocyte cytolysis of NK-sensitive and NK-resistant target cells. Mononuclear cells depleted of adherent cells were cultured for 4 or 10 days, with or without 100 units/mL recombinant human IL-2. Cultures were harvested and cells washed thoroughly prior to use as effectors at varying $E:T$ ratios with ^{111}In-labeled K562 (NK-sensitive) or Daudi (NK-resistant) target cells. After 4 days of culture with increasing concentrations of AZT, IL-2-treated lymphocytes demonstrated modest and variable reductions in cytolytic activity against K562 and Daudi compared to IL-2 cultures without AZT (Fig. 1, A and B). Following 10 days of culture under similar conditions, lymphocytes demonstrated a more striking AZT concentration-related inhibition of cytolysis (Fig. 1, C and D). Parallel cultures of lymphocytes with the same AZT concentrations but in the absence of exogenous IL-2 appeared to be similarly inhibited for cytolysis of K562 (data not shown). These non-activated lymphocytes mediated very low levels (≦10% lysis) of cytotoxicity against NK-resistant Daudi or Raji cells, and lysis of K562 by control lymphocytes was reduced markedly following 9–10 days of culture without exogenous IL-2, so that effects of AZT were difficult to assess in control lymphocyte cultures.

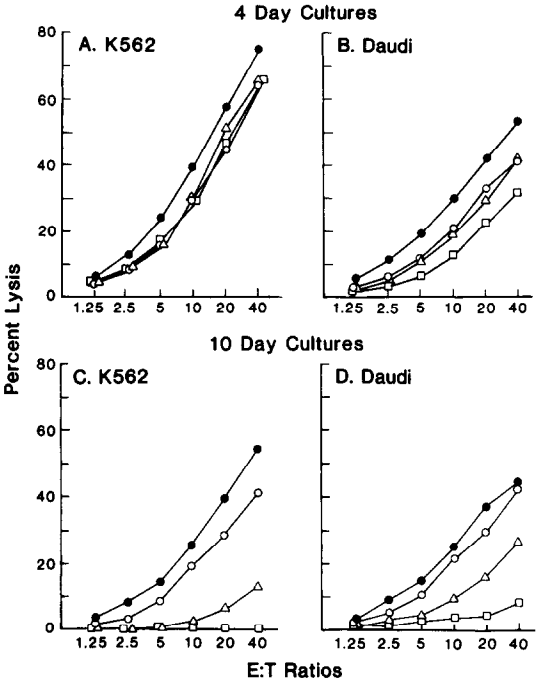

Fig. 1. Effect of AZT added to IL-2 cultures of lymphocytes. Lymphocytes were cultured with 100 units/mL IL-2, either without AZT (●) or with AZT at 1 μM (○), 10 μM (△) or 100 μM (□) final concentration. After 4 days (A and B) or 10 days (C and D) of culture, lymphocytes were assayed for cytotoxicity against K562 (A and C) and Daudi (B and D) targets. Results shown are from a single representative experiment (N = 5 with different donor lymphocytes).

We next examined whether AZT concentrations that reduced lymphocyte IL-2-dependent cytolytic activity were cytotoxic to lymphocytes. As shown in Table 1, 10-day cultured lymphocytes demonstrated only minor AZT concentration-related reductions in total cell numbers and cell viability that seemed unlikely to be directly responsible for the extensive reductions in cytolytic activity observed (e.g. Figs. 1 and 2); it should be pointed out that all effector suspensions were prepared based upon numbers of viable cells. Furthermore, lymphocyte cell surface antigen expression in 10-day lymphocyte cultures was not altered dramatically by AZT up to 100 μM (Table 2), suggesting that individual major subpopulations of lymphocytes were not

Table 1. Viability of cultured lymphocytes

	Cells × 10^{-6}			
	Control cultures		IL-2 cultures	
	Total	Viable	Total	Viable
No AZT	11.7 ± 2.0	10.8 ± 1.9	12.0 ± 1.5	11.0 ± 1.8
1 μM AZT	9.6 ± 1.1	9.2 ± 0.9	12.4 ± 2.5	11.9 ± 2.3
10 μM AZT	8.9 ± 1.5	8.9 ± 2.1	9.9 ± 92.1	9.0 ± 2.0
100 μM AZT	10.7 ± 5.8	9.2 ± 5.0	11.3 ± 5.3	9.9 ± 5.3

Results are means ± SD for four independent 10-day cultures of lymphocytes. Viable cells are those that excluded Trypan blue.

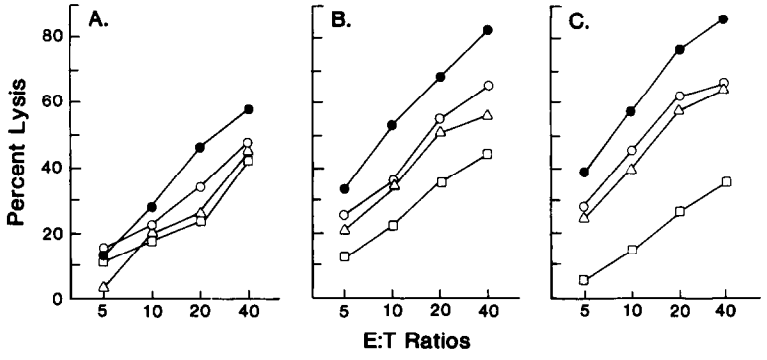

Fig. 2. Effect of IL-2 concentration on AZT suppression of lymphocyte cytolytic activity. Lymphocytes were cultured for 4 days with IL-2 at 1 (A), 10 (B) or 100 (C) units/mL and the co-addition of AZT at 1 (○), 10 (△) or 100 (□) μM or no AZT (●). Harvested lymphocytes were assayed for cytotoxicity against Daudi targets. Results shown are from a single representative experiment (N = 3 with different donor lymphocytes).

Table 2. Cultured lymphocyte cell surface markers

	Percent positive cells						
	Leu-4	Leu-3	Leu-2	Leu-17	Leu-19	DR	IL-2R
Control cultures							
No AZT	96	79	21	64	22	28	28
1 μM AZT	97	79	20	61	21	20	22
10 μM AZT	97	80	19	60	23	23	26
100 μM AZT	97	81	22	60	23	24	28
IL-2 cultures							
No AZT	99	73	27	86	15	23	12
1 μM AZT	99	75	26	85	15	20	12
10 μM AZT	99	75	28	85	16	24	13
100 μM AZT	99	76	25	73	20	27	14

Lymphocytes were cultured for 10 days with or without IL-2 and the indicated AZT additions. Harvested lymphocytes were analyzed for cell surface markers by indirect immunofluorescence flow cytometry. Results are percent positive cells in the lymphocyte gate out of 10,000 gated events for a single representative experiment (N = 3).

preferentially eliminated or suppressed during culture with AZT.

Lymphocytes cultured with lower amounts of exogenous IL-2 were also cytolytically activated and similarly inhibited by AZT (Fig. 2). However, low-concenration IL-2 lymphocyte cultures did not appear to be more sensitive to AZT than high-concentration IL-2 cultures; indeed, lymphocytes

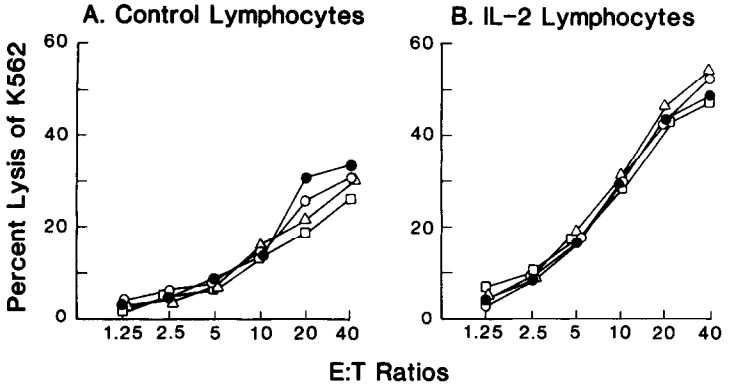

Fig. 3. Effect of AZT addition to cytotoxicity assays with preactivated lymphocytes. Lymphocytes were cultured for 2 hr with or without 100 units/mL IL-2 and then assayed for cytotoxicity against K562 targets in the presence of 1 (○), 10 (△) or 100 (□) μM AZT or no AZT (●). Results shown are from a single representative experiment (N = 3 with different donor lymphocytes).

Table 3. Interferon-γ production in lymphocyte cultures

	Interferon-γ (units/mL)			
	Control cultures		IL-2 cultures	
	Day 5	Day 11	Day 5	Day 11
No AZT	0.75	1.0	40	43
1 μM AZT	0.95	0.95	36	48
10 μM AZT	0.55	1.0	34	51
100 μM AZT	0.95	1.2	42	72

Results are the means of duplicate determinations by radioimmunoassay of cell-free supernatants from a single representative experiment (N = 3).

cultured with 1 unit/mL IL-2 (Fig. 2A) appeared to be less sensitive to AZT suppression than lymphocytes cultured with 100 units/mL IL-2 (Fig. 2C). These results suggest that AZT does not directly interfere with IL-2 binding to lymphocyte IL-2 receptors. To test another possibility, that AZT may directly interfere with lymphocyte cytolytic mechanisms, AZT was added to the cytotoxicity assay media with K562 targets and lymphocytes previously cultured with IL-2 (in the absence of AZT). As shown in Fig. 3, AZT did not suppress cytolysis by previously activated lymphocytes. Furthermore, AZT did not reduce the level of interferon-γ production by IL-2-cultured lymphocytes as measured by radioimmunoassay of interferon-γ in culture supernatants (Table 3).

Finally, we compared the effects of AZT with two other nucleoside anti-viral drugs, ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine] and acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) (Fig. 4). Equimolar concentrations of ganciclovir and acyclovir did not mimic the suppressive effects of AZT on 10-day IL-2-cultured lymphocyte cytolytic activity, although 100 μM acyclovir resulted in modest levels of inhibition.

DISCUSSION

The present study demonstates that AZT exerts a negative effect on the cytolytic activity of long-term IL-2-cultured (10 day) lymphocytes. This effect was not correlated with reduced lymphocyte viability or with major alterations in lymphocyte surface phenotypes. Further, IL-2-stimulated release of interferon-γ by lymphocytes was not suppressed by AZT. Thus, the effects of AZT on these cultured lymphocytes appears to be relatively selective. Parallel experiments with other anti-viral nucleoside drugs indicated that AZT was uniquely potent in its inhibition of lymphocyte cytolytic activity.

Mitsuya *et al.* [12] previously reported that *in vitro* immune functions of normal lymphocytes are "basically" not affected by AZT at concentrations between 1 and 50 μM, but their data did suggest an AZT concentration-dependent inhibition of [^{3}H]uridine incorporation into cellular RNA by antigen-stimulated lymphocyte cultures. AZT did not reduce the viability of cloned helper T cells cultured with IL-2 for 14 days, results similar to those reported here, but lymphocyte cytolytic activity was not assessed [12]. Lyerly *et al.* [10] also observed a slight inhibition of lymphocyte proliferative responses to specific antigen in the presence of 1 μM AZT.

More profound effects of AZT on lymphocyte activity *in vitro* were reported by assaying human T lymphocyte pokeweed mitogen-stimulated DNA synthesis via cellular [^{3}H]thymidine incorporation [13]. It must be noted, however, that AZT is a congener of thymidine, and we have reported previously that thymidine can reverse the cytopathic effects of AZT as well as its anti-viral activity [8]. Thus, the results of those authors [13] may actually reflect competitive inhibition of cell utilization of the tritiated pyrimidine by a molar excess of the analogue AZT. There have been additional apparently conflicting reports regarding the effects of AZT on phytohemagglutinin (PHA) mitogen-stimulated growth of human lymphocytes [8, 9, 12, 14]. However, it should be emphasized that

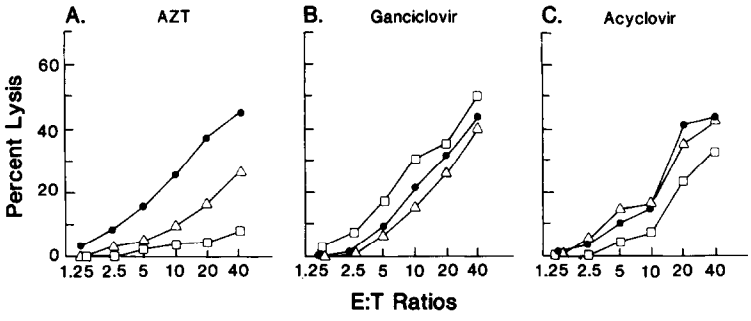

Fig. 4. Comparison of AZT with other anti-viral drugs. Lymphocytes were cultured for 10 days with 100 units/mL IL-2 and the co-addition of AZT (A), ganciclovir (B) or acyclovir (C) at final concentrations of 10 (△) or 100 (□) μM drug or no drug (●), prior to cytotoxicity assays against Raji targets. Results shown are from a single representative experiment (N = 3 with different donor lymphocytes).

the present work used IL-2-cultured lymphocytes and that the function primarily assessed was lymphocyte cytolytic activity. Whereas mitogenic responses in our IL-2-cultured lymphocytes were not directly measured in this study, data on cell numbers, viability and surface phenotype (Tables 1 and 2) indicate that inhibition of cell growth was not a major contributing factor in the reduction of lymphocyte cytolysis following long-term exposure to AZT. Indeed, our studies were designed from the outset to analyze AZT concentrations that were minimally cytopathic.

These collective data support the hypothesis that AZT, at effective anti-viral concentrations but below cytotoxic concentrations, may affect a restricted subset of cellular functions such as those operative in the IL-2-activated lymphocytes studied here, and that this may be mediated via selective effects independent of cellular proliferation. Elucidation of the molecular mechanisms involved in the suppression of lymphocyte cytolytic activity by AZT could potentially impact on the long-term treatment of immunocompromised patients with AZT and related drugs.

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