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Effect of 3′-Azido-3′-Deoxythymidine and 2′,3′-Dideoxyinosine on Establishment of Human Immunodeficiency Virus Type 1 Infection in Cultured CD8$^+$ Lymphocytes

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Several groups have shown that peripheral CD8$^+$ lymphocytes can be infected with human immunodeficiency virus type 1 (HIV-1), resulting in noncytopathic infection and persistent production of viral particles. We studied the ability of 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI) to inhibit the establishment of HIV-1 infection in CD8$^+$ cells that were derived from cultures of peripheral blood lymphocytes exposed to both virus and drug. In situ infection of CD8$^+$ cells was demonstrated by double flow cytometry analysis by using both anti-glycoprotein 120 (anti-gp120) and anti-CD8 monoclonal antibodies. At higher concentrations of drug (e.g., 0.4 μM AZT), the production of viral particles was inhibited for over 2 months, as assessed by p24 antigen levels in the culture medium. We also performed a time course experiment to determine whether HIV-1 infection of CD8$^+$ cells would be affected by treatment of peripheral blood lymphocytes with AZT or ddI for different intervals following exposure to virus. Quantitative PCR revealed that 0.4 μM AZT, added as late as 24 h after infection, interfered with the formation of proviral DNA in CD8$^+$ cells. Both HIV-1 load and the production of progeny virions by CD8$^+$ cells, as monitored by reverse transcriptase activity in culture fluids, were inhibited by both AZT and ddI in a dose-dependent manner.

Although CD4$^+$ T cells are the principal targets of human immunodeficiency virus (HIV), other cell types can be infected as well, often noncytopathically, including B lymphocytes (6, 16), thymocytes (12), monocytes/macrophages (11, 17), dendritic cells (13), eosinophils (10), neurons (14), hepatocytes (4), colorectal cells (1), and CD34$^+$ bone marrow-derived precursor cells (9). Several groups have also described the productive and persistent infection of CD8$^+$ lymphocytes by HIV type 1 (HIV-1) (5, 7, 8, 15). Since HIV-1 tropism is not as highly restricted as was first thought, the efficiency of a given antiviral agent should be tested by using a variety of target cell types.

The subject of HIV-1 infection of CD8$^+$ lymphocytes may be especially important, since such cells have been shown to constitute an important component of the active cytotoxic T-lymphocyte response against HIV-1-infected targets (18, 21). Indeed, clinical trials involving the genetically modified CD8$^+$ cytotoxic T-lymphocyte response in adoptive immunotherapy are in progress. In addition, CD8$^+$ lymphocytes may suppress viral replication through secretion of soluble factors (3, 22). Therefore, it is important to understand to what extent antiviral chemotherapy might successfully antagonize HIV-1 replication in CD8$^+$ lymphocytes. Here we report on the effects of 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI) in interfering with the establishment of HIV-1 infection in CD8$^+$ cells.

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MATERIALS AND METHODS

Cells and viruses. Peripheral blood mononuclear cells were purified from the blood of healthy HIV-1-seronegative donors by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation and were stimulated for 3 days with 10 μg of phytohemagglutinin (Difco, Detroit, Mich.) per ml in RPMI 1640 medium (Gibco Corp., Toronto, Ontario, Canada) supplemented with 10% heat-inactivated fetal calf serum (FCS)–2 mM L-glutamine–250 U of penicillin per ml–250 μg of streptomycin per ml–10 U of interleukin-2 (Boehringer Mannheim, Montreal, Canada) per ml as described previously (15). AZT and ddI were gifts from Burroughs Wellcome, Inc., Montreal, Quebec, Canada, and Bristol-Myers Squibb, Montreal, Quebec, Canada, respectively, and were not toxic for either peripheral blood lymphocytes (PBLs) or CD8$^+$ cells at the concentrations used. The III_B strain of HIV-1 (HIV-III_B) was grown on the highly susceptible MT-4 T-cell line. The 50% tissue culture infectious dose ($TCID_{50}$) was determined six days after infection by means of an indirect immunofluorescence assay by using an anti-p24 monoclonal antibody (MAb) as described previously (2), as were reverse transcriptase (RT) assay, indirect immunofluorescence assay, and viral p24 core antigen enzyme-linked immunosorption assay (2).

Infection of peripheral blood lymphocytes. Nonadherent cells were gently decanted from stimulated peripheral blood mononuclear cells to establish enriched populations of PBLs. PBLs were infected with HIV-III_B at a multiplicity of infection of 0.02 $TCID_{50}$ per cell for 3 h at 37°C in 0.5 ml. Control cultures of uninfected PBLs were also maintained in these experiments. Cells were washed twice to remove unadsorbed viral particles and were transferred at a concentration of 5×10^5 cells per ml to a 75-cm^2 flask in the presence of drug, which

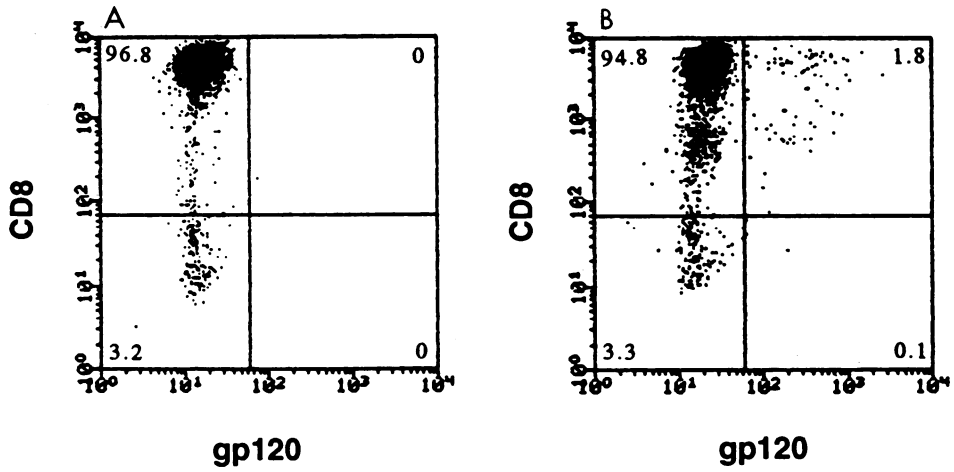

FIG. 1. Two-color flow cytometry analysis of in situ infection of peripheral CD8+ cells not infected (A) or infected (B) with HIV-1.

was subsequently added at twice-weekly intervals at each medium change. Cultures were incubated at 37°C under 5% CO_2, and cell viability was monitored by trypan blue exclusion. At 7 days after infection, both infected and uninfected PBLs were enriched for CD8+ lymphocytes by positive selection. Briefly, PBLs (10^7 cells) were first labeled with 50 μl of anti-CD8 MAb (Leu2a; Becton Dickinson, Mountain View, Calif.) for 30 min on ice. Cells were washed twice in phosphate-buffered saline (PBS) and were then incubated at 37°C for 1 h in 0.5 ml of 7.0×10^7 biomagnetic particles coated with goat anti-mouse immunoglobulin G specific for the Fc component (Advanced Magnetics Inc., Cambridge, Mass.). Thereafter, CD8+ lymphocytes were sorted out by submitting labeled cultures to an appropriate magnetic field. Biomagnetic particles were subsequently removed from the cultures, which were maintained at 37°C. Cultures enriched for CD8+ cells were monitored over time by flow cytometry for the presence of contaminating CD4+ lymphocytes; fewer than 2% of such cells were present after cell separation. Each experiment was performed at least three times.

Quantitative HIV-1 DNA amplification. The oligonucleotide primers used for HIV-1 DNA detection were derived from the nucleotide sequence of the ARV-2 isolate of HIV-1 and correspond to a conserved glycoprotein 41 (gp41) region of the *env* gene. The primers were SK68 (5′-AGCAGCAGGAAG CACTATGC-3′, sense) and SK69 (5′-CCAGACTGTGAGT TGCAACAG-3′; antisense) (19). The amplified product resulting from the SK68-SK69 pair of primers is a 140-bp fragment. Detection of human beta-globin DNA was accomplished with primers at positions 14 to 33 (5′-ACACAACT GTGTTCACTAGC-3′, sense) and 123 to 104 (5′-CAACT TCATCCACGTTCACC-3′; antisense). When used in conjunction, these two primers formed an amplified product of 110 bp that could easily be distinguished from the HIV-1-specific fragment.

Only the sense primers were end-labeled with ^{32}P, while antisense primers were unlabeled; 20 and 30 ng of each type, respectively, were incorporated into the reaction mixture. Each reaction mixture contained 200 μM (each) the four de-

TABLE 1. Effects of AZT and ddI on percentages of CD8+ and gp120+ cells and levels of p24 antigen in culture fluids[a]

Drug	Drug concn (μM)	% CD8+ and gp120+ cells[b]	p24 antigen concn (ng/ml)[b]
None		1.9 ± 0.3	9.2 ± 1.6
AZT	0.004	2.1 ± 0.4	6.5 ± 1.4
AZT	0.04	0.3 ± 0.1	3.2 ± 0.5
AZT	0.4	0	0
ddI	1	2.2 ± 0.4	10.6 ± 2.3
ddI	10	0.6 ± 0.1	3.4 ± 0.6
ddI	90	0.2 ± 0.005	0.8 ± 0.1

[a] PBLs were infected with HIV-1 27 days previously, as described in the text. AZT and ddI were added to PBLs, prior to the establishment of CD8+ subcultures, from the end of the 2-h viral adsorption period.

[b] Values are means ± standard deviation and are based on three replicate cultures.

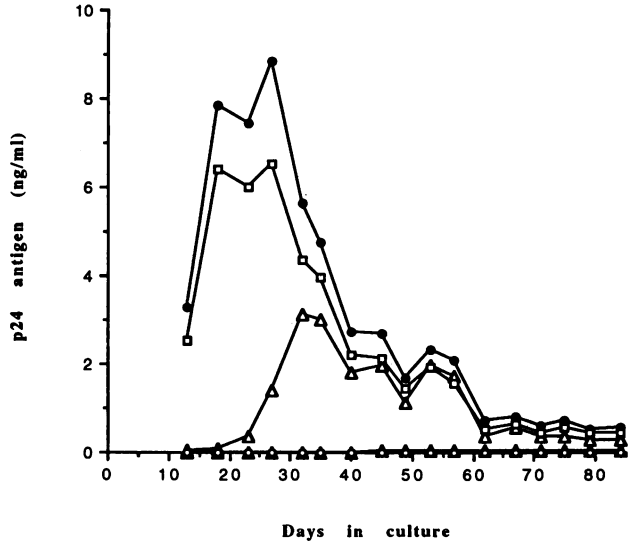

FIG. 2. Effect of AZT on HIV-1 replication in cultured CD8+ cells. Phytohemagglutin-P-activated PBLs were either uninfected (○) or infected with HIV-1 strain III_B at 0.02 $TCID_{50}$ per cell for 2 h. The cultures were maintained in the absence of AZT (●) or in the presence of AZT at 0.004 μM (□), 0.04 μM (△), or 0.4 μM (▲). On day 8 following infection, CD8+ cells were purified by using immunomagnetic particles and were maintained in culture in the presence or absence of AZT as described above.

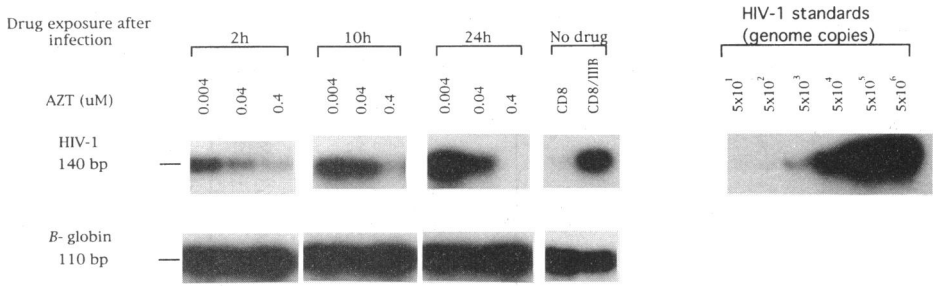

FIG. 3. Effect of AZT on proviral DNA burden in $CD8^+$ lymphocytes. Freshly stimulated PBLs were treated with AZT at various times (2, 10, 24 h) after infection with HIV-1 strain III_B at 0.01 $TCID_{50}$ per cell. $CD8^+$ cells were purified 11 days later, and total DNA was extracted on day 15. The HIV-1 genomic copy number was estimated by quantitative PCR by using pHXB-2, which contains a full-length molecular clone of HIV-1 digested with *Xho*I. The beta-globin (β-globin) gene served to standardize the amount of DNA used for amplification.

oxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 3 U of *Taq* DNA polymerase (Bethesda Research Laboratories).

DNA amplification was performed by PCR, and estimates of the numbers of genomic copies of HIV-1 in $CD8^+$ lymphocytes were determined by quantitative PCR as described previously (15). Products were analyzed by electrophoresis on 8% nondenaturing polyacrylamide gels, dried, and visualized by phosphor imaging (model GS250; Bio-Rad, Mississauga, Ontario, Canada).

Two-color flow cytometry analysis of in situ-infected $CD8^+$ cells. PBLs (10^5) were harvested and washed once with cold PBS containing 5% FCS and 0.015% sodium azide (PBS-FA). Cells were then pelleted and incubated on ice for 30 min with 3 to 5 μl of an anti-gp120 MAb (0.5β; AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases). The cells were then incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Becton Dickinson) for 30 min on ice. The second MAb, an anti-CD8 MAb directly labeled with phycoerythrin (Leu2a; Becton Dickinson), was added to the cells for another 30 min on ice. After each step, the cells were washed twice in cold PBS-FA. At the end of the procedure, cells were fixed in 0.5% formaldehyde and analyzed with a FACScan flow cytometer (Becton Dickinson).

RESULTS

HIV-1 infection of $CD8^+$ cells. The purities of our $CD8^+$ cultures were determined by means of two-color flow cytometry analysis by using both anti-CD4 and anti-CD8 MAbs. In each experiment, $CD8^+$ cells represented more than 95% of the cell population, which contained fewer than 2% contaminating $CD4^+$ cells (data not shown). Samples of 17-day cultures of untreated HIV-1-infected and uninfected $CD8^+$ cells were double-labelled with MAbs against CD8 and the viral envelope glycoprotein gp120. We found that 1.8% of cells expressed both of these markers at the surface membrane (Fig. 1). More than 96% of cells were positive for CD8.

Effects of AZT and ddI on establishment of HIV-1 infection in $CD8^+$ cells. To determine whether the virus produced by $CD8^+$ cells was infectious, we exposed H9 cells (10^6) to clarified supernatants of untreated, infected $CD8^+$ cultures that were harvested at different times after the establishment of infection. The H9-cell targets became positive by indirect immunofluorescence assay for p24 antigen at 5 to 16 days after infection (data not shown).

To assess the effect of AZT on the establishment of HIV-1 infection in $CD8^+$ cells, we measured the levels of p24 antigen in culture fluids of these cells. Drug was present from the time of infection of PBLs and was maintained throughout the steps leading to the preparation of $CD8^+$ cultures and thereafter. In the absence of AZT treatment of PBLs or at the lowest concentration of AZT used (0.004 μM), a peak in the p24 antigen level occurred at 27 days after infection (Fig. 2). In contrast, 0.04 μM AZT caused delays in the generation of p24 antigen in $CD8^+$ culture fluids, and 0.4 μM AZT virtually abolished p24 antigen production over a 3-month period.

The fractions of $CD8^+$ and gp120-positive ($gp120^+$) cells in 27-day PBL-derived cultures that were exposed to either AZT or ddI at a variety of concentrations are presented in Table 1. We found that untreated cultures contained approximately 2% doubly positive ($CD8^+$ $gp120^+$) cells. In contrast, when PBLs were exposed to either 0.4 μM AZT or 90 μM ddI, the numbers of such cells declined significantly, along with the

TABLE 2. Temporal dependence of inhibitory effects of AZT and ddI on RT levels in culture fluids of HIV-1-inoculated cultures

Drug	Time of drug addition (h)	Drug concn (μM)	RT activity (% of untreated control) on the following (day in culture):			
			7	15	20	27
AZT	2	0.004	ND[a]	25.4	52.1	14.3
		0.04	ND	22.2	7.9	15.1
		0.4	ND	2.5	2.6	1.2
	10	0.004	ND	32.0	60.5	42.1
		0.04	ND	29.6	10.5	16.6
		0.4	ND	3.4	3.3	2.8
	24	0.004	ND	46.9	6.7	77.8
		0.04	ND	37.0	21.1	33.2
		0.4	ND	2.6	2.9	3.0
ddI	2	1	78.2	67.1	81.2	107.1
		10	21.7	1.9	1.5	0.5
		90	17.4	1.6	1.8	0.9
	10	1	147.8	107.1	106.1	81.4
		10	26.1	8.1	27.7	54.6
		90	21.7	1.2	1.5	0.7
	24	1	121.7	90.9	96.4	92.2
		10	21.7	26.7	102.8	78.0
		90	8.6	1.0	1.8	0.4

[a] ND, not done.

levels of p24 antigen in the culture fluids. Both effects occurred in a concentration-dependent fashion.

Effect of AZT on HIV-1 load in CD8+ lymphocytes. Quantitative DNA amplification revealed that provirus levels were affected both by the dose of AZT used and the time of addition of drug to virus-inoculated cultures of PBLs. Increases in the AZT concentration caused notable reductions in the levels of detectable proviral DNA (Fig. 3). This was also influenced by the time of drug addition following infection. When 0.004 and 0.04 μM AZT were added at 10 and 24 h after infection, proviral DNA levels in CD8+ cells were 2.6 and 5.2 times higher, respectively, than in similar cultures established from PBLs to which drug was added after 2 h (Fig. 3). The number of HIV-1 DNA copies remained low for all time points studied when a concentration of 0.4 μM AZT was used.

Effect of delayed drug treatment on HIV-1 replication in CD8+ lymphocytes. To assess the effect of a delay in drug addition, CD8+ cells were treated with either AZT or ddI at 10 or 24 h after exposure of parental PBL cultures to HIV-1. At the highest AZT concentration used (0.4 μM), we found that no RT activity could be detected over 27 days, even when drug was added only after 24 h (Table 2). In contrast, a clear temporal dependence was apparent when either 0.04 or 0.004 μM AZT was used, with higher levels of enzyme activity associated with delays in the addition of the compound. Similar findings were obtained when ddI was used to treat PBLs or derivative, infected CD8+ populations (Table 2). However, in this instance, analyses were also performed just prior to enrichment for CD8+ populations at day 7 after infection. It can be seen that moderate to high concentrations of drug (10 or 90 μM) interfered with the levels of RT activity otherwise obtained (no drug or 1 μM ddI), regardless of whether compound was added at the end of the adsorption period or after 10 or 24 h.

DISCUSSION

The ability of AZT and ddI to inhibit HIV-1 RT activity and subsequent viral replication in each of CD4-expressing lymphocyte cell lines, primary cultures of T-helper lymphocytes, and CD4-positive monocytic cell cultures is well established (23). However, little attention has been paid to the ability of nucleosides, including AZT and ddI, to antagonize HIV-1 replication or the establishment of infection in other cell types. It is well established that CD8+ lymphocytes can be infected with HIV-1 under appropriate circumstances (5, 7, 8, 15). Accordingly, we investigated the ability of AZT to inhibit viral replication in these cells.

We showed that both the formation of the HIV-1 proviral genome and HIV-1 replication in populations of CD8+ cells can be inhibited by treatment of PBLs with each of AZT and ddI. Furthermore, these effects were demonstrated to occur in a dose-dependent fashion. We also showed that the time of drug addition following infection plays a significant role with regard to outcome in terms of both viral load and expression of viral products. Notably, a delay in the addition of drug from the end of the adsorption period to later times gave rise to increased production of RT and p24 antigen except for studies which involved the highest dose of AZT. In parallel experiments, we showed that neither AZT nor ddI was toxic for either unfractionated PBLs or CD8+ cells at the concentrations used.

Aspects of our experimental design were clearly not ideal because of the difficulty of accomplishing a de novo infection of enriched CD8+ populations (5, 7, 15). Therefore, it was possible to assess only the establishment of HIV-1 infection in CD8+ cells that were derived from HIV-1-infected PBL populations that included CD4+ lymphocytes. Subsequent transmission of HIV-1 from infected CD4+ cells to CD8+ lymphocytes may have been through cell-to-cell contact (5, 7, 15). Thus, we cannot clearly distinguish between direct inhibition of HIV-1 replication in CD8+ cells and inhibition in CD4+ cells, with a consequent reduction in the numbers of infected CD8+ lymphocytes. Presumably, both AZT and ddI are able to antagonize viral RT activity and the establishment of infection in CD8+ cells following such interactions. This notion is substantiated by the finding that differences in RT levels were present after 7 days, but prior to the establishment of CD8+ subcultures. In addition, delays in the addition of AZT and/or ddI to HIV-1-inoculated cultures of PBLs led to increased levels of RT activity and p24 antigen levels in culture fluids of CD8+ lymphocytes and to higher numbers of CD8+ and gp120+ cells. This presumably reflects the fact that higher numbers of CD4+ cells became infected under such circumstances, leading, in turn, to ultimate infection of higher numbers of CD8+ targets. In current studies, we are attempting to monitor the numbers of CD8+ and gp120+ cells in both treated and untreated samples by serial flow cytometry.

Others have shown that CD8+ lymphocytes can acquire HIV-1 in tissue culture through cell-to-cell transmission from CD4+ cells (7). Nevertheless, infectious viruses produced by CD8+ cells do not easily spread to uninfected CD8+ cells, probably because of the absence of the CD4 receptor on the latter (7, 15). In our study, the percentage of infected CD8+ cells remained low throughout the culture period after removal of CD4+ cells from unfractionnated PBLs. Once infected, CD8+ cells can apparently express HIV-1 proteins without expressing a cytopathic effect. The ability of AZT and ddI to prevent infection of CD8+ cells might be due, in part, to their effects on HIV-1 replication in CD4+ T lymphocytes.

A previous study showed that CD8+ CD4− lymphocytes from HIV-1-seropositive individuals express HIV-1 antigens (20). The ability of peripheral CD8+ cells to persistently produce infectious HIV-1 emphasizes the need to study further the effects of antiretroviral drugs in the context of non-CD4+ cells, particularly in chronically HIV-1 infected targets.

The role of CD8+ cells in helping to curb HIV-1 replication levels in CD4+ lymphocytes and to act as cytotoxic T lymphocytes for HIV-infected targets is well established (3, 18, 21, 22). Although few CD8+ cells are likely at any time to be infected by HIV-1 it will be important to assess whether such an infection might compromise the anti-HIV-1 activities usually displayed by such cells. Both in this context and from the standpoint of limiting HIV-1 spread from infected CD8+ cells, the effects of antiviral nucleosides in the CD8+ population merit increased attention.

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