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Sanctuary Growth of Human Immunodeficiency Virus in the Presence of 3′-Azido-3′-Deoxythymidine

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Human immunodeficiency virus (HIV) resistance to the nonnucleoside reverse transcriptase inhibitors emerges very rapidly under selection in culture and in patients. In contrast, zidovudine (3′-azido-3′-deoxythymidine [AZT])-resistant HIV generally emerges in patients only after more-prolonged therapy. Although HIV can be cultured from many patients shortly after the initiation of AZT treatment, characterization of the virus that is cultured generally indicates that it is sensitive to AZT. To initiate an evaluation of the mechanisms contributing to early HIV breakthrough in the presence of AZT and other nucleoside analogs, we have utilized replication-defective HIV encoding reporter genes. These recombinant HIV allow a quantitative analysis of a single cycle of infection. Results with these defective HIV indicate that early infection in the presence of AZT often results from the infection of a cell which is refractory to the antiretroviral effects of AZT. Characterization of a cell line derived from one such cell has demonstrated decreased accumulation of AZT triphosphate, increased phosphorylation of thymidine to thymidine triphosphate, and increased levels of thymidine kinase activity. In addition, AZT inhibition of replication-competent HIV infection is also significantly impaired in this cell line. Attempts to detect and characterize the mechanisms responsible for early viral infection after initiation of AZT therapy may result in the development of new strategies for prolonged suppression of viral infection prior to the emergence of drug-resistant virus.

When antiretroviral therapy is initiated for a patient with human immunodeficiency virus (HIV) infection, there is generally a virologic response characterized by declining viremia and antigenemia (5, 7, 19, 20, 25). Unfortunately, the currently available antiretroviral agents which have undergone clinical evaluation have only limited benefit, in that most patients will ultimately have evidence of worsening disease and increasing viral burden. This progression often occurs in association with the emergence of drug-resistant HIV. For example, most patients who are treated with zidovudine (3′-azido-3′-deoxythymidine [AZT]) will have initial evidence of improvement of the clinical and laboratory parameters of HIV infection (7, 19). The duration of this benefit varies from patient to patient and is likely to be disease stage related (21). Ultimately, however, most patients will have progressive disease and genotypic or phenotypic evidence of the appearance of AZT-resistant HIV (9, 12). Since clinical failure and the appearance of virus with high-level resistance to AZT both coincide with evidence of increasing levels of viremia and changes in viral tropism, it has been difficult to ascribe the clinical failure solely to the development of AZT resistance (2, 11). Nevertheless, it seems likely that AZT resistance ultimately contributes to the clinical failure seen in most patients receiving prolonged AZT therapy.

While the development of virus-encoded drug resistance may contribute to the limited efficacy of currently used antiretroviral agents, it cannot explain all of the in vitro and in vivo phenomena associated with viral replication in the presence of an antiretroviral agent. For example, many patients will have continuing evidence of viral replication after the initiation of AZT therapy; however, the isolated virus will remain sensitive to AZT when it is analyzed in tissue culture (7, 19). In contrast, high-level HIV resistance to many of the nonnucleoside reverse transcriptase inhibitors develops very rapidly in culture and in patients (13, 16, 22, 23). Some of these differences may relate to the complexity and prevalence of viral variants harboring pre-existing drug resistance mutations prior to the application of the selective pressure. However, some of the differences may be due to cellular heterogeneity in the uptake or metabolism of the antiretroviral agents. In other words, each cell population may have some cells that are refractory to the antiviral effects of the drug. This would allow a subset of the cellular population to be successfully infected by genetically drug-sensitive HIV in the presence of the antiviral drug. Depending on the prevalence of drug-resistant HIV in the initial population, the relative rates of replication of drug-resistant and drug-sensitive virus, and the percentage of cells refractory to the antiviral effects of the drug, different patterns of viral breakthrough would emerge. Notably, the nonnucleoside reverse transcriptase inhibitors do not undergo cellular metabolism, and cellular effects of uptake or metabolism may be less likely in this setting. This is consistent with the observation that virus-encoded drug resistance to the nonnucleoside reverse transcriptase inhibitors develops very rapidly under selection in tissue culture and in patients. In fact, the rapid development of resistance in patients suggests that the blood and plasma compartment of the virus is subjected to selective drug pressure. The presence of HIV but lack of AZT-resistant HIV early after the initiation of AZT suggest that a component of this viral pool may be capable of averting selective drug pressure in vivo. Continued viral replication in cells in which AZT is an ineffective antiretroviral agent could conceivably result in

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the continued growth of virus that is sensitive to AZT (sanctuary growth). An increase in the number of these cells over time could also alter viral growth kinetics in the presence of AZT without the emergence of virus with high-level AZT resistance. Therefore, many mechanisms may contribute to the inability of an antiviral agent to completely suppress HIV infection. Viral growth in the presence of the nonnucleoside reverse transcriptase inhibitors appears to be due to the rapid selection of genetically resistant virus. In contrast, genetic viral drug resistance does not appear to be the major mechanism contributing to early viral growth in the presence of AZT.

A previous report has detailed the use of recombinant HIV-encoding reporter genes to analyze viral breakthrough infection in the presence of antiretroviral agents (26). In that study, to determine the prevalence of viral variants spontaneously resistant to the nonnucleoside reverse transcriptase inhibitor TIBO R82150, HeLa-T4 cells were infected in the presence of drug with replication-defective HIV-gpt (18, 26) or HIV-LacZ (26). The recombinant virus used for these infections was produced by infection of cell lines containing an integrated copy of the defective recombinant virus with replication-competent HIV. The replication-competent HIV provided the necessary gene products to rescue the defective virus. The prevalence of viral variants containing mutations encoding resistance to TIBO R82150 was reflected by the prevalence of recombinant viruses capable of infecting HeLa-T4 cells in the presence of TIBO R82150. The presence of reporter genes in the recombinant viruses allowed for a quantitative analysis of a single cycle of infection on a single cell basis.

In this report, a similar analysis of early events after HIV infection in the presence of AZT and other nucleoside analogs is reported. In these studies, cells were infected with recombinant HIV, and infection was monitored by reporter gene expression. By using two recombinant retroviruses as well as a replication-competent stock of HIV, it has been possible to ascribe a significant component of early HIV infection in the presence of AZT to the infection of cells which are refractory to the antiretroviral effects of AZT.

MATERIALS AND METHODS

Construction of recombinant proviral DNA. The HIV construct encoding LacZ has been described elsewhere (26). It contains the *lacZ* gene driven by a simian virus 40 (SV40) promoter inserted into a large deletion in the HIV genome extending from the 5′ end of the *pol* gene to the 3′ end of the *env* gene. The HIV-gpt and HXB2env plasmids were kindly provided by Kathleen Page (University of California, San Francisco, Calif.) (18). The HIV-gpt plasmid contains an HXB2 provirus into which an SV40 promoter *gpt* (*Escherichia coli* guanine phosphoribosyltransferase) gene was inserted into the *env* region. The HXB2env plasmid contains the HXB2 gp160 gene driven by an SV40 promoter.

Production of plasmid-derived recombinant retroviruses. All transfections and cell cultures were performed in an approved facility using biosafety level 3 techniques. Plasmid DNA cotransfections into COS cells were performed as described by Page et al. (18). Supernatants from COS cells were collected 40 h after transfection and assayed for infectious recombinant HIV-LacZ by inoculation of 2×10^5 HeLa-T4 cells with 0.1 ml of filtered (0.45-μm-pore-size filter) supernatant. The cells were stained for beta-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) 48 h after infection as described elsewhere (26, 27). To assay for infectious recombinant HIV-gpt, the infected cells were split 1:10 into *gpt*-selective medium as described elsewhere (26). Medium changes were performed every 3 days, and colonies were counted 10 to 14 days postinfection after staining with 1% crystal violet in 10% formalin.

Cell lines containing defective HIV-gpt and HIV-LacZ. The H9/HIV-gpt cell line and the HeLa-T4/HIV-LacZ cell line were prepared and used as previously described (26). Rescue of defective retroviruses from the H9/HIV-gpt cell line and the HeLa-T4/HIV-LacZ cell line was performed as previously described (26). Following each rescue infection, the resultant titer of HIV-LacZ or HIV-gpt was determined and the inoculum used to infect HeLa-T4 cells was adjusted, depending on the number of infectious events to be analyzed.

High-performance liquid chromatography (HPLC) analysis of clones. Cell lines were incubated with [^{3}H]thymidine or [^{3}H]AZT for 4 h. Dried methanol extracts of the clones were redissolved in 60 μl of distilled water and centrifuged to remove undissolved material. Twenty microliters of the sample was injected and separated on a Rainin Hydropore anion exchange column (10 by 100 mm). The nucleotides were eluted from the column with a linear gradient of potassium phosphate (5 mM to 1 M [pH 4.0]) at a flow rate of 1 ml/min. The samples were collected (0.5 ml), mixed with 5 ml of Packard scintillation fluid, and quantitated with a liquid scintillation counter. Phosphorylated derivatives of thymidine and AZT were identified with authentic standards.

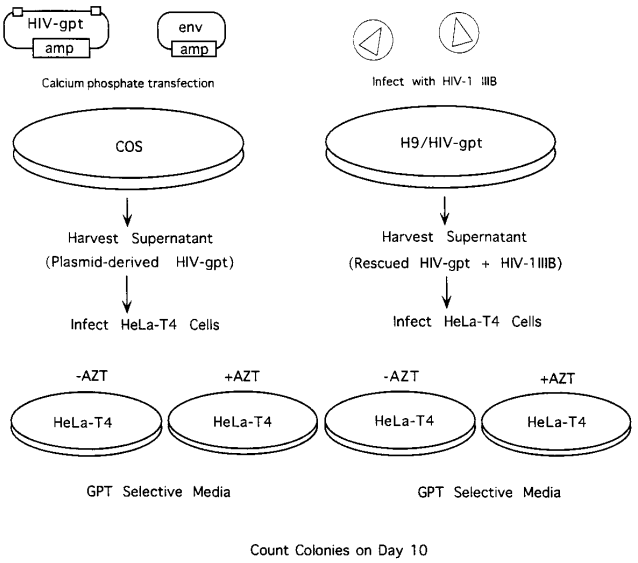


FIG. 1. Schematic representation of the production of recombinant HIV-gpt by COS cell transfection or rescue from the H9/HIV-gpt cell line.

Cytotoxicity assay. AZT-mediated cytotoxicity in cells persistently refractory to the antiviral effects of AZT (R116) and in cells sensitive to the antiviral effects of AZT (HT4, S pool, and S1) was assayed (14). Triplicate wells of 24-well plates containing 3×10^4 cells were cultured in the absence or presence of various concentrations of AZT. Three days later, drug cytotoxicity was quantitated with a standard 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay in which the uptake and metabolism of MTT by cells was measured (14). The amount of formazan produced in 2 h was determined by dissolving the product in 100% dimethyl sulfoxide and then measuring the A_{570}.

Northern (RNA) blot analysis. Total RNAs from S1 and R116 cells were extracted as described previously (4). Equal amounts of total RNAs were electrophoresed on an agarose gel containing 1% formaldehyde and were blotted onto a nylon membrane. The RNAs were hybridized with a ^{32}P-labeled human thymidine kinase probe (3). The labeled bands were visualized by autoradiography and were quantitated with a Molecular Dynamics Personal Densitometer.

Thymidine kinase assay. Thymidine kinase activities in cell lines sensitive and resistant to AZT were determined. Cellular extracts of S1 and R116 cells were prepared according to Sherley and Kelly (24) and were assayed for thymidine kinase activity as described by Lee and Cheng (10). The protein concentration of each extract was determined with Bio-Rad protein reagent.

RESULTS

HIV-gpt infection of cells in the absence and presence of AZT. HeLa-T4 cells were infected with a recombinant HIV, HIV-gpt, in the presence or absence of 10 μM AZT (Fig. 1). Two separate populations of HIV-gpt were utilized for these infections. One population of HIV-gpt was produced in COS cells by cotransfection of the HIV-gpt plasmid with a plasmid encoding the HXB2 *env* gene. The infectious virions produced by this cotransfection have little genetic diversity in that they are produced from products encoded by plasmids in COS cells. The second population of HIV-gpt was genetically more diverse, since it was produced by rescue from the H9/HIV-gpt cell line with replication-competent HIV-1_{IIIB} that had been propagated in culture (26). After infection, the HeLa-T4 cells were placed in *gpt* selective medium, and the number of colonies developing by day 10 was used as an indicator of the number of cells initially infected in the absence or presence of

TABLE 1. Frequency of HIV-gpt colony formation in the presence and absence of AZT[a]

Source of HIV-gpt	No. of colonies		Frequency
	Without AZT	With AZT	
Plasmid-derived (COS cells)	3.1×10^4	16	5.2×10^{-4}
Rescue with HIV-1	1.8×10^4	9	5.0×10^{-4}

[a] HIV-gpt produced in COS cells by transfection with plasmids is compared with HIV-gpt produced by rescue from the H9/HIV-gpt cell line after infection with HIV-1_{IIIB} (see Fig. 1).

10 μM AZT. As can be seen from Table 1, the values for frequency of colony formation after infection in the presence of AZT were similar ($\sim 5 \times 10^{-4}$) for the two preparations of HIV-gpt. This similarity is very distinct from the results of infections performed in the presence of a nonnucleoside reverse transcriptase inhibitor, TIBO R82150. In those studies, the prevalence of infection with the COS cell-derived virus was 20-fold lower than infection with HIV-gpt rescued by replication-competent virus (26). Since the HIV-gpt produced in COS cells would not be expected to be genetically diverse, this relatively high rate of infection in the presence of AZT was not likely the result of virus-encoded AZT resistance. Similarly, the absence of more-prevalent infection in the presence of AZT when HIV-gpt was produced by rescue with a propagated stock of replication-competent HIV implies that true genetic resistance was not detected in these experiments. These data suggest that other mechanisms contribute to this early viral breakthrough in the presence of AZT.

Identification of cells refractory to the antiviral effects of nucleoside analogs. To characterize further the mechanism(s) of viral infection accounting for the high frequency of colony formation after infection in the presence of 10 μM AZT, the experiment depicted in Fig. 2 was performed. HeLa-T4 cells were infected with HIV-gpt (prepared in COS cells) in the absence or presence of AZT. Infected cells were selected in *gpt*-selective medium, and colonies were isolated and expanded into cell lines. Twelve cell lines developing after infection in the presence of AZT were further characterized. To determine if these cell lines were refractory to the antiretroviral effects of AZT, they were infected with HIV-LacZ in the presence of 10 μM AZT. Three days after infection, the cells were stained with X-Gal to detect β-galactosidase activity. A total of 9 of these 12 cell lines behaved like wild-type HeLa-T4 cells, with complete inhibition of infection in the presence of AZT. However, 3 of these cell lines demonstrated greater than 50% of control infection (−AZT) despite the presence of 10 μM AZT. These cell lines were labeled persistently resistant to the antiretroviral effects of AZT.

Infection of these persistently resistant cell lines with replication-competent HIV confirmed the relative inefficacy of AZT in these cells. For example, a clinically relevant concentration of 0.1 μM AZT was much less effective in inhibiting HIV-1_{IIIB} in the persistently resistant cell line than in the control cells (Fig. 3). No such cells resistant to the antiviral effects of AZT were obtained when colonies derived from HIV-gpt infections in the absence of AZT were studied (see Table 2).

None of the persistently resistant cell lines were cross-resistant to the antiretroviral effects of ddI or ddC. Interestingly, cells with persistent resistance to AZT showed partial cross-resistance to the antiretroviral effects of 50 μM 3′-dideoxy-2′,3′-didehydrothymidine.

A further characterization of this cellular cross-resistance is currently under way. In addition to this evaluation for cellular cross-resistance, it was possible to use a similar experimental protocol to demonstrate the independent selection of cells refractory to the antiretroviral effects of a variety of other

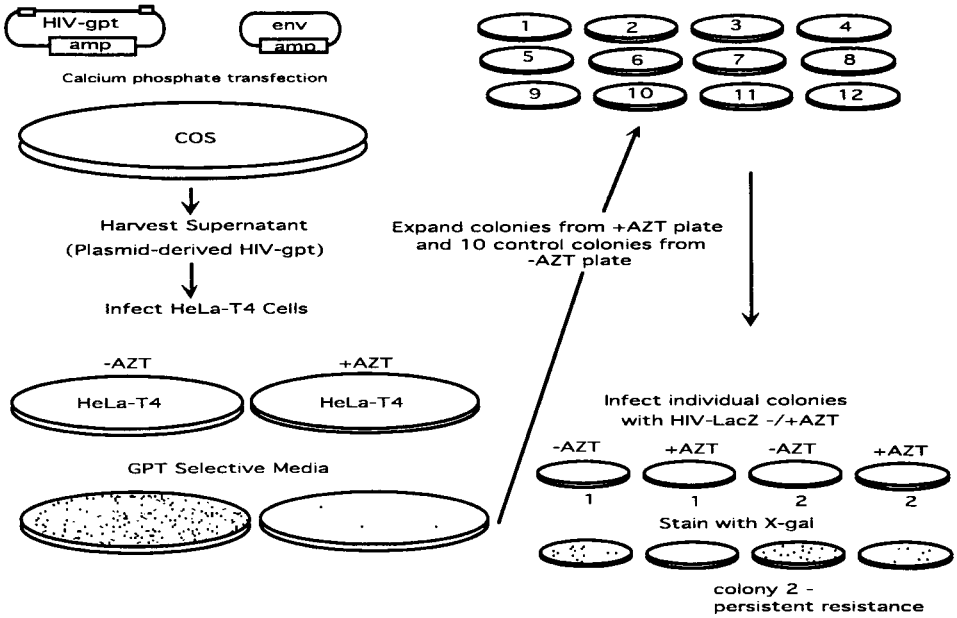


FIG. 2. Schematic representation of the analysis of colonies arising after COS cell derived HIV-gpt infection of HeLa-T4 cells in the presence of 10 μM AZT. Twelve such colonies were expanded and infected with HIV-LacZ in the presence or absence of 10 μM AZT. Ten control colonies derived from HIV-gpt infection of HeLa-T4 cells in the absence of AZT were studied in parallel. Persistent cellular resistance was defined by a high-level infection with HIV-LacZ in the presence of AZT, as shown for colony number 2. HIV-LacZ contains the *lacZ* gene driven by an SV40 promoter inserted into a large deletion in the HIV genome extending from the *pol* gene to the 3′ end of the *env* gene. HIV-LacZ production has been previously described (26).

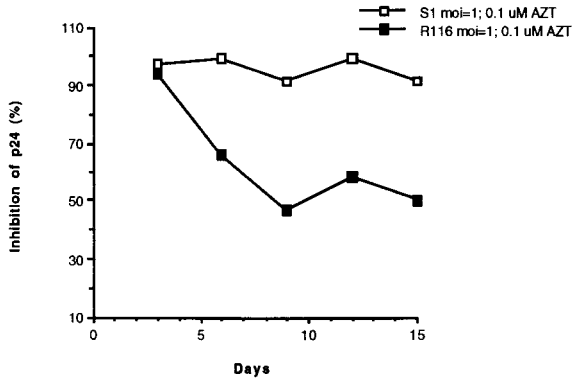
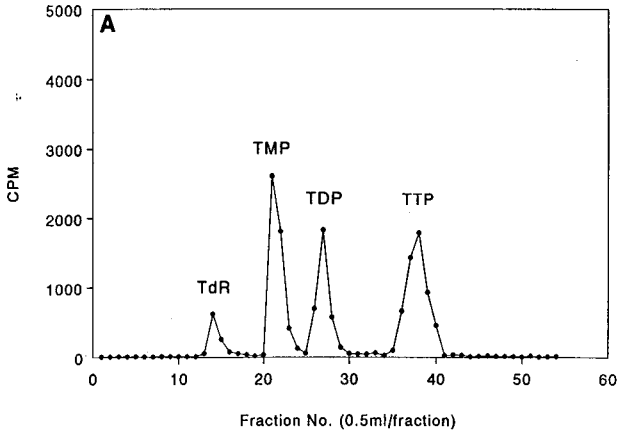


FIG. 3. Infection of a clone of HeLa-T4 cells persistently resistant to the antiviral effects of AZT (clone R116) and a control clone (S1 [cell line derived from a clone of HeLa-T4 cells infected with HIV-gpt in the absence of AZT]) with replication-competent HIV-1_{IIIB} in the presence of 0.1 μM AZT. The amounts of p24 were assayed, compared with those for a control infection in the absence of AZT, and plotted as a function of time. p24 values in the absence of AZT were 1,857 ± 104 ng/ml for S1 and 1,717 ± 113 ng/ml for R116. moi, multiplicity of infection.

nucleoside analogs (Table 2). In contrast, no cells persistently resistant to the antiretroviral effects of the nonnucleoside reverse transcriptase inhibitor TIBO R82150 could be selected by identical techniques. These results indicate that HeLa-T4 cells have subpopulations of cells that are independently refractory to the antiretroviral effects of a variety of nucleoside analogs.

Comparison of thymidine and AZT phosphorylation in isolated clones. To initiate an analysis of the mechanisms responsible for this cellular resistance, a persistently resistant cell line was compared with a control cell line obtained by HIV-gpt infection in the absence of AZT. Each of these cell lines was incubated with [^{3}H]thymidine, and thymidine metabolites were assayed by HPLC. As shown in Fig. 4, the persistently resistant cell line (R116) had a greater phosphorylation of thymidine into thymidine triphosphasphate (TTP) than the nonresistant cell line (S1). An identical experiment with [^{3}H]AZT indicated a nearly twofold reduction in AZT triphosphate (AZTTP) in R116 cells compared with S1 cells (Table 3). Therefore, a component of the resistance may be related to a diminished AZT TP/TTP ratio. These results suggest that alterations in nucleotide metabolism may underlie some of the differences between these cell lines. To further characterize the basis for these differences, thymidine kinase mRNA levels and thymidine kinase activities in the two cell lines were compared. Although there were no differences in the thymidine kinase mRNA levels by a Northern blot analysis, the R116 cell line had three times more thymidine kinase activity than the S1 cell line (Fig. 5).



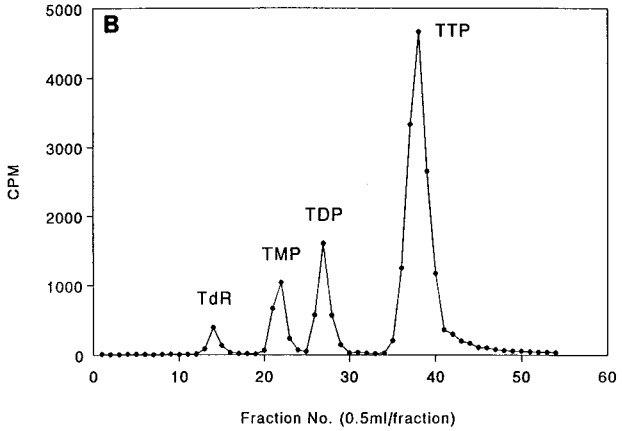


FIG. 4. Thymidine (TdR) metabolism. HPLC analysis of clones obtained after infection of HeLa-T4 cells with HIV-gpt in the presence and absence of AZT (with 0.1 μM thymidine). (A) S1 cell line derived from HeLa-T4 cells after infection with HIV-gpt in the absence of AZT; (B) R116 cell line, which was persistently resistant to the antiviral effects of AZT. The earliest peak represents thymidine and the subsequent peaks represent phosphorylated metabolites.

Tolerance of the clones for very high concentrations of AZT. In additional studies of these cell lines, the tolerance for high concentrations of AZT was tested. As shown in Fig. 6, the persistently resistant cell line (R116) was much more tolerant

TABLE 2. Colony formation and persistent resistance after HeLa-T4 infection with plasmid derived HIV-gpt (produced in COS cells) in the presence of high doses of antiretroviral agents[a]

Drug	No. of colonies	No. of persistently resistant colonies	Frequency
Control (no drug)	8,800	0/10	0
AZT	12	3/12	3.4×10^{-4}
D4T	50	Not done	
DDI	16	2/16	2.3×10^{-4}
TIBO	3	0/3	0

[a] Concentrations of the antiretroviral agents were as follows: AZT, 10 μM; DDI, 50 μM; D4T, 50 μM; and TIBO, 1 μM.

TABLE 3. Concentration of phosphorylated AZT metabolites in the persistently resistant (R116) and sensitive (S1) cell lines[a]

Clone	Metabolite (pmol/10^6 cells)			
	AZT	AZTMP	AZTDP	AZTTP
S1	0.0206 (12.2)	0.1212 (71.6)	0.0116 (6.9)	0.0158 (9.3)
R116	0.0155 (7.7)	0.1575 (78.6)	0.0193 (9.6)	0.0083 (4.2)

[a] Pool sizes were determined by incubation of cells with [^{3}H]AZT for 4 h, followed by cellular extraction and HPLC. The numbers in parentheses are the percentages of total radioactive species in each pool.

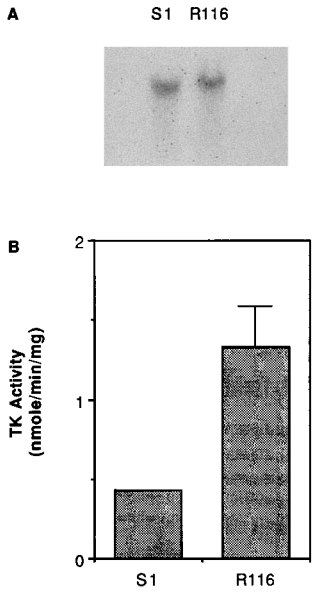


FIG. 5. Comparison of thymidine kinase (TK) mRNA levels (A) and enzyme activities (B) in cell lines sensitive and persistently resistant to the antiretroviral effects of AZT. The mRNA levels of S1 and R116 were 8,390 and 8,500 densitometry units, respectively. Thymidine kinase activities were based on three independent experiments performed in triplicate.

of high concentrations of AZT. The cytotoxic concentration of AZT that killed 50% of a variety of control cell lines was approximately 100 μM. In contrast, the cytotoxic concentration of AZT that killed 50% of the persistently resistant clone R116 was greater than 1 mM. This implies that the mechanisms that protect HIV from AZT in the resistant cell lines also protect these cell lines from the cytotoxic effects of even higher concentrations of AZT. This demonstrates another AZT-related difference among these clones that are derived from the same parental cell line.

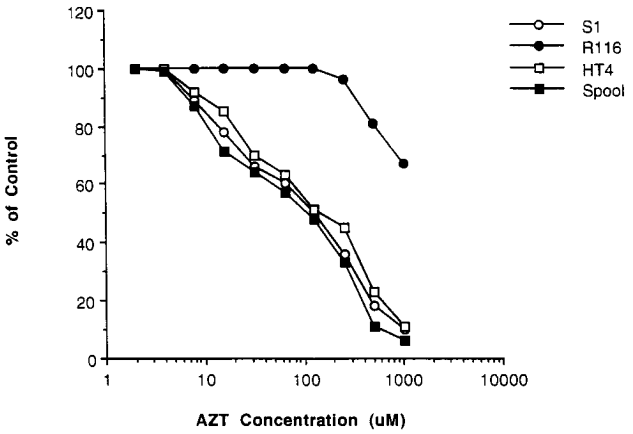


FIG. 6. Cellular toxicity of AZT. The cell lines were grown in the presence of the indicated concentrations of AZT. Cellular toxicity values were then determined for cells persistently refractory to the antiviral effects of AZT (R116) and for cells sensitive to the antiviral effects of AZT (HT4, S pool, and S1) using a standard MTT assay. S pool was a pool of colonies derived from HIV-gpt infection of HeLa-T4 cells in the absence of AZT. HeLa-T4 is the parental cell line.

DISCUSSION

The studies described above indicate that sanctuary growth of HIV may occur in the presence of AZT and that early in treatment, cellular resistance may make a large contribution to viral breakthrough. In fact, there was no quantitative difference in HIV breakthrough when HIV-gpt prepared by transfection in COS cells was compared with HIV-gpt produced by rescue with replication-competent HIV. This suggests that a large part of early infection in the presence of AZT may be a consequence of cellular effects. We have detected at least two types of such sanctuary growth. A total of 9 of the 12 cell lines analyzed did not have persistent resistance to the antiviral effects of AZT and may have had epigenetic alterations such as those that might occur at specific points in the cell cycle. In contrast, 3 of the 12 cell lines had persistent resistance to the antiviral effects of AZT, with both recombinant and replication-competent HIV. In studies with replication-competent HIV, virtually complete inhibition of the infection of control cells was obtained with a concentration of AZT that reduced viral production in a persistently resistant clone only by 50%.

These cell lines refractory to the antiviral effects of AZT are likely to have specific alterations that render AZT less effective. Metabolic studies suggest that some of this resistance may be due to differences in nucleotide metabolism resulting in a reduction of AZTTP in the resistant cells. It will be important to further characterize and define the mechanisms responsible for cellular resistance, because reversal of this resistance may greatly reduce viral burden and delay the outgrowth of virus with genetic resistance. It is important to emphasize that the cells that were detected as refractory to the antiviral effects of AZT were exposed to AZT only for a short period of time. There was no preselection of cells prior to infection with the recombinant viruses.

It will be important to extend these observations to primary blood mononuclear cells. Unfortunately, only low levels of expression of the recombinant viruses used in these studies can be achieved in primary blood mononuclear cells. Furthermore, it is difficult to obtain prolonged culture of clones of primary cells infected with these viruses. Therefore, efforts to perform similar experiments with primary cells will require modifications in the experimental approach. These studies are currently under way.

Recent reports on nucleotide pool sizes in resting as opposed to stimulated blood mononuclear cells and different cell lines derived from different blood cell lineages have demonstrated marked differences that might translate into variable efficacies of nucleoside analogs within populations of blood cells (6, 15). Furthermore, other investigators have grown cells in high concentrations of AZT for prolonged periods of time and demonstrated the selection of cells with reduced levels of thymidine kinase activity (17). Additional data about metabolic differences occurring in the lymphocytes of patients treated with prolonged courses of AZT also suggest that cellular resistance contributes to HIV breakthrough (1). Thus, cellular resistance is likely to contribute to viral breakthrough during an in vivo infection, and multiple mechanisms may contribute to cellular resistance. The prevalence of resistant cells detected in a single cell line in our studies raises interesting speculation concerning the prevalence of similar resistant cells during an in vivo infection involving multiple cell types.

Earlier studies with recombinant viruses indicated that there is a high prevalence of genetically TIBO-resistant HIV in an unselected HIV population. As a consequence of this high prevalence and the lack of cellular metabolism for TIBO, genetically resistant virus is rapidly selected in vivo and in vitro.

In contrast, AZT is metabolized in cells, a subpopulation of which is refractory to the antiretroviral effects of AZT. Early growth of nongenetically resistant virus can occur in these sanctuary cells (cellular resistance). With continued growth, there is amplification of pre-existing (or emerging) viral variants with genetic resistance because the truly resistant virus can infect any suitable target cell, not just cells in which AZT is ineffective. This gives a relative growth advantage to the genetically resistant virus. Subsequent additional mutations or recombination events may result in viruses with multiple mutations. The initial cellular resistance may allow a population of nonresistant or partially resistant virus to replicate, providing a pool of virus in which additional mutations and recombination events can occur. Reversal of cellular resistance could conceivably delay, or even prevent, the outgrowth of highly resistant virus with multiple mutations by not allowing nonresistant or partially resistant virus (with single mutations) to replicate.

The objective of the studies presented in this report is to establish that HIV sanctuary growth in the presence of AZT can be an important component of HIV breakthrough early after the initiation of AZT treatment. Additional studies will be necessary to determine the relevance of cellular resistance in in vivo infection and the contribution of cellular resistance to the emergence of genetically resistant virus.

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REFERENCES

1. **Avramis, V. I., R. Kwock, M. M. Solorzano, and E. Gomperts.** 1993. Evidence of in vitro development of drug resistance to azidothymidine in T-lymphocytic leukemia cell lines (Jurkat E6-1/AZT-100) and in pediatric patients with HIV-1 infection. J. Acquired Immune Defic. Syndr. **6:**1287–1296.
2. **Boucher, C. A., J. M. Lange, F. F. Miedema, et al.** 1992. HIV-1 biological phenotype and the development of zidovudine resistance in relation to disease progression in asymptomatic individuals during treatment. AIDS **6:**1259–1264.
3. **Bradshaw, H. D., and P. L. Deininger.** 1984. Human thymidine kinase gene: molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. Mol. Cell. Biol. **4:**2316–2320.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Fischl, M. A., D. D. Richman, M. H. Grieco, et al.** 1987. The efficacy of azidothymidine AZT in the treatment of patients with AIDS and the AIDS related complex. A double blind placebo controlled trial. N. Engl. J. Med. **317:**185–191.
6. **Gao, W.-Y., T. Shirasaka, D. G. Johns, S. Broder, and H. Mitsuya.** 1993. Differential phosphorylation of azidothymidine, dideoxycytidine and dideoxyinosine in resting and activated peripheral blood mononuclear cells. J. Clin. Invest. **91:**2326–2333.
7. **Johnston, M. I., and J. J. McGowan.** 1992. Strategies and progress in the development of antiretroviral agents, p. 357–372. *In* V. T. DeVita, S. Hellman, and S. A. Rosenberg (ed.), AIDS: etiology, diagnosis, treatment and prevention. J. B. Lippincott Company, Philadelphia.
8. **Larder, B. A., K. E. Coates, and S. D. Kemp.** 1991. Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. J. Virol. **65:**5232–5236.
9. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
10. **Lee, L.-S., and Y.-C. Cheng.** 1976. Human deoxythymidine kinase. J. Biol. Chem. **251:**2600–2604.
11. **Mayers, D. L.** 1993. Clinical significance of in vitro zidovudine resistance. Third workshop on viral resistance, Gaithersburg, Md.
12. **Mayers, D. L., F. E. McCutchan, B. E. Sanders, et al.** 1992. Characterization of HIV isolates arising after prolonged zidovudine therapy. J. Acquired Immune Defic. Syndr. **5:**749–759.
13. **Mellors, J., G. Dutschman, G. Im, E. Tramontano, S. R. Winkler, and Y. C. Cheng.** 1992. In vitro selection and molecular characterization of HIV-1 resistant to nonnucleoside inhibitors of reverse transcriptase. Mol. Pharmacol. **41:**446–451.
14. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
15. **Mukherji, E., J. L. S. Au, and L. E. Mathes.** 1994. Differential antiviral activities and intracellular metabolism of 3′-azido-3′-deoxythymidine and 2′,3′-dideoxyinosine in human cells. Antimicrob. Agents Chemother. **38:**1573–1579.
16. **Nunberg, J. H., W. A. Schleif, E. J. Boots, J. A. O'Brien, J. C. Quintero, J. M. Hoffman, E. A. Emini, and M. E. Goldman.** Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. J. Virol. **65:**4887–4892.
17. **Nyce, J., S. Leonard, D. Canupp, S. Schulz, and S. Wong.** 1993. Epigenetic mechanisms of drug resistance: drug induced DNA hypermethylation and drug resistance. Proc. Natl. Acad. Sci. USA **90:**2960–2964.
18. **Page, K. A., N. Landau, and D. R. Littman.** 1990. Construction and use of a human immunodeficiency virus vector for analysis of of viral infectivity. J. Virol. **64:**5270–5276.
19. **Richman, D. D.** 1992. Antiretroviral therapy: azidothymidine and other deoxynucleoside analogues, p. 373–387. *In* V. T. DeVita, S. Hellman, and S. A. Rosenberg (ed.), AIDS: etiology, diagnosis, treatment and prevention. J. B. Lippincott, Philadelphia.
20. **Richman, D. D., M. A. Fischl, M. H. Grieco, et al.** 1987. The toxicity of azidothymidine in the treatment of patients with AIDS and AIDS-related complex: a double blind placebo controlled study. N. Engl. J. Med. **317:**192–197.
21. **Richman, D. D., J. M. Grimes, and S. W. Lagakos.** 1990. Effect of stage of disease and drug dose on zidovudine susceptibilities of isolates of human immunodeficiency virus. J. Acquired Immune Defic. Syndr. **3:**743–746.
22. **Richman, D. D., C. K. Shih, I. Lowy, et al.** 1991. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc. Natl. Acad. Sci. USA **88:**11241–11245.
23. **Saag, M. S., E. A. Emini, and O. L. Laskin.** 1993. A short term clinical evaluation of L-697,661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase. N. Engl. J. Med. **329:**1065–1072.
24. **Sherley, J. L., and T. J. Kelly.** 1988. Human cytosolic thymidine kinase. J. Biol. Chem. **263:**375–382.
25. **Spector, S. A., C. Kennedy, and J. A. McCutchan.** 1989. The antiviral effect of zidovudine and ribavirin in clinical trials and the use of p24 antigen as a virologic marker. J. Infect. Dis. **159:**822.
26. **Strair, R. K., D. J. Medina, C. J. Nelson, T. Graubert, and J. W. Mellors.** 1993. Recombinant retroviral systems for the analysis of drug-resistant HIV. Nucleic Acids Res. **21:**4836–4842.
27. **Strair, R. K., C. J. Nelson, and J. W. Mellors.** 1991. Use of recombinant retroviruses to characterize the activity of antiretroviral compounds. J. Virol. **65:**6339–6342.