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Anti-HIV-1 activity of 3′-azido-3′-deoxythymidine (AZT) in primary mononuclear phagocytes

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Summary

Conflicting data have been reported on ability of 3′-azido-3′-deoxythymidine (AZT) to protect mononuclear phagocytes from HIV-1 infection. We compared the antiviral potency of AZT in three types of primary human mononuclear phagocytes: peripheral blood monocytes, monocyte-derived macrophages (in vitro differentiated) and alveolar macrophages (in vivo differentiated). To establish highly-productive virus infection, purified cells (>99%) from healthy donors were challenged with the macrophage-tropic HTLV-III$_{Ba-L}$ strain at input multiplicities ranging from 0.05 to 20 TCID$_{50}$ per cell. AZT (0.1 nM–10 μM) was added immediately after infection and either continued for the duration of the experiment or stopped 1–7 days after infection. The kinetics of HIV-1$_{Ba-L}$ replication were assessed by measuring p24 antigen production on days 4–28 post-infection. Continuous treatment with AZT reproducibly inhibited viral replication in a concentration-dependent manner in all three cell types. The IC$_{90}$ of AZT was 0.04 μM in blood monocytes, 0.009 μM in monocyte-derived macrophages, and 0.0001 μM in alveolar macrophages (mean of 3–4 donors for each cell type). AZT was not cytotoxic at < 10 μM as assessed by cell viability, cell protein, and interferon-γ-activated H_2O_2-release. In experiments in which AZT treatment was stopped after infection, viral replication resumed after a lag of 7–14 days and increased exponentially toward control levels. This occurred despite initial inhibition of virus production to below the limit of p24 detection (~ 50 pg/ml).

These results indicate that AZT is a potent inhibitor of HIV-1 replication in primary mononuclear phagocytes regardless of the stage of cell differentiation, and that AZT is most active in tissue (alveolar) macrophages. AZT does not

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irreversibly block infection of mononuclear phagocytes, however, as viral replication resumes after removal of AZT.

AIDS; HIV-1; 3′-Azido-3′-deoxythymidine; AZT; Zidovudine; Mononuclear phagocyte; Monocyte; Macrophage; Alveolar macrophage

Introduction

Cells of the monocyte-macrophage lineage are believed to play an important role in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection (Gartner et al., 1986; Roy et al., 1988; Meltzer et al., 1990). Macrophages are the predominant cells infected with HIV-1 in brain and spinal cord (Stoler et al., 1986; König et al., 1986; Wiley et al., 1986; Eilbott et al., 1989). HIV-1-infected macrophages have also been demonstrated in lung (Chayt et al., 1986), lymph nodes (Baroni et al., 1986), liver (Housset et al., 1991), muscle (Chad et al., 1990), and skin (Tschachler et al., 1987). These virus-infected cells are thought to constitute an important cellular reservoir of HIV-1 and to contribute to organ dysfunction, particularly in brain and spinal cord (Meltzer et al., 1990).

The 2′,3′-dideoxynucleosides (ddNs), including 3′-azido-3′-deoxythymidine (AZT), are potent inhibitors of HIV-1 replication in proliferating T-cells (Mitsuya et al., 1985; Mitsuya and Broder, 1986). Conflicting data have been reported on the ability of ddNs to inhibit HIV-1 replication in mononuclear phagocytes. Richman et al. (1987) reported that AZT and other ddNs did not inhibit HIV-1 replication in monocyte-derived macrophages at concentrations up to 100 μM. In contrast, Perno et al. (1988) reported $>95\%$ inhibition of HIV-1 replication in fresh peripheral blood monocytes treated with 0.5 μM AZT. Two-fold higher concentrations of AZT (~ 1.0 μM) were necessary to inhibit HIV-1 replication in 5-day cultured monocyte-derived macrophages (Perno et al., 1988). In both of these studies, the macrophages that were used as target cells for HIV-1 were derived from blood monocytes after in vitro differentiation (Richman et al., 1987; Perno et al., 1988). It is uncertain whether these in vitro differentiated cells are representative of tissue macrophages in vivo. Thus, the antiviral activity of AZT in tissue macrophages has not been clearly defined.

In the present study, therefore, we compared the anti-HIV-1 activity of AZT in three types of primary mononuclear phagocytes: peripheral blood monocytes, monocyte-derived macrophages (in vitro differentiated) and alveolar macrophages (in vivo differentiated).

Materials and Methods

Materials

A 10-mM stock solution of AZT (Sigma Chemical, St. Louis, MO) was prepared in phosphate-buffered saline (PBS), stored at −20°C and diluted in medium immediately prior to use. The concentration of the stock solution was verified spectrophotometrically using an extinction coefficient of 11650 M^{-1} at 267 nm. Recombinant human interferon-gamma (rIFNγ) with a specific activity of 2×10^7 units/mg was a gift from Genentech Inc. (South San Francisco, CA).

Cell culture

All tissue culture reagents were screened for the presence of contaminating endotoxins with the limulus amebocyte lysate assay (sensitivity 10–100 pg/ml) as described (Mellors et al., 1991). Peripheral blood mononuclear cells (MNC) were isolated from healthy, HIV-1 seronegative donors by density-gradient centrifugation on ficoll-Na diatrizoate (Sigma). MNC were resuspended to the desired concentration in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD), supplemented with 2 mM L-glutamine, 25 IU/ml penicillin, 25 μg/ml streptomycin and 10% heat-inactivated human AB serum (Advanced Biotechnologies, Silver Spring, MD).

To purify peripheral blood monocytes (PBM), 1.5×10^7 MNC in 2.0 ml of medium were seeded into 3.8-cm^2 wells of 12-well plates (Costar, Cambridge, MA) and incubated at 37°C for 2 h. Non-adherent cells were removed by several washes with PBS. To obtain monocyte-derived macrophages (MDM), 3.8-cm^2 wells were seeded with 5×10^6 MNC suspended in 2.0 ml of medium. After 7 days of culture, the non-adherent cells were removed by several washes with PBS. The attached cells were then cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) for an additional week to allow complete differentiation into macrophages (Gartner et al., 1986; Mellors et al., 1991; Gan et al., 1991).

Alveolar macrophages (AM) were obtained from healthy, non-smoking donors by bronchoalveolar lavage (BAL). All volunteers were free from pulmonary or systemic illness as previously described (Rankin et al., 1989). The total number of BAL cells retrieved, differential cell counts and macrophage viability (>95%) were similar to those reported for other normal volunteers studied in our laboratory (Rankin et al., 1989). The lavage fluid was strained through a sterile gauze filter to remove particulate matter and centrifuged at 500 × *g* for 10 min at 24°C. The cell pellet was then resuspended in RPMI 1640/10% AB serum. About 4×10^5 alveolar lavage cells were seeded into 1.0-cm^2 wells of 48-well plates (Costar) and incubated for 2 h at 37°C. The non-adherent cells were removed by several washes with PBS. Complete medium changes were performed twice weekly for all cell types.

Cell purity

The purity of the cell populations was assessed by modified Wright-Giemsa staining (Diff-Quik, American Scientific Products, McGaw Park, IL) and alpha-naphthyl acetate esterase staining (Sigma, Kit No. 91-A). At the time of HIV-1 infection, >99% of the cells exhibited characteristic monocyte/macrophage morphology in Wright-Giemsa stained preparations. MDM and AM were >99% non-specific esterase positive by alpha-naphthyl acetate staining, while PBM were ~92% esterase-positive on the day of infection, but became >99% esterase positive by day 5 of culture.

Virus

The macrophage-tropic HIV-$1_{Ba\text{-}L}$ strain (kindly provided by S. Gartner, M. Popovic, and R. Gallo, NCI, Bethesda, MD) was propagated in MDM (Gartner et al., 1986). The supernatant from stock virus cultures was removed weekly, centrifuged (400 × *g* for 10 min at 4°C) to remove cellular debris and stored at −70°C. Stock virus infectivity was determined by triplicate end-point dilution in MDM using p24 antigen production as the indicator of viral replication (assayed on day 28 post-inoculation). The 50% tissue culture infectious dose ($TCID_{50}$) was calculated with the Reed-Muench (1938) equation for interpolation. The infectivity titer of stock virus preparations ranged from $10^{4.5}$ to $10^{6.5}$ $TCID_{50}$/ml.

Infection of cells

PBM and AM were infected with HIV-1_{BaL} within 3 h of cell purification. MDM were infected on day 14 of culture. To infect MDM and PBM, stock virus at a multiplicity of infection (MOI) of 0.05–10 $TCID_{50}$ per cell was adsorbed for 3 h. For AM, pilot experiments demonstrated that virus adsorption for 18–24 h at an MOI of 10–20 $TCID_{50}$ per cell was necessary to establishe productive infection comparable to that observed in MDM and PBM. After virus adsorption, cells were washed 4 times with PBS to remove the inoculum. Samples of the final wash were found to be free of residual inoculum (<50 pg/ml of p24 antigen).

HIV-1 replication

Culture supernatants were sampled twice weekly, treated with 0.5% Triton X-100, and stored at −20°C. The concentration of p24 antigen in culture supernatants was measured with an enzyme immunoassay according to the manufacturer's instructions (Abbott Laboratories, N. Chicago, IL). Samples from the same experiment were run in large batch assays to minimize the potential effect of inter-assay variability on results.

Assays of drug cytotoxicity

Drug-induced cytotoxicity was assessed in uninfected cells after 14 days (PBM and MDM) or 28 days (AM) of culture. Three parameters were measured: cell viability, cell mass (protein), and response to activation with

rIFNγ. The number of viable cells/well (trypan blue exclusion) was determined with an inverted microscope using an optical grid at 200× magnification (Olympus Corp., Lake Success, NY). At least 10 random fields were counted per well. The total cell mass was estimated by determining the amount of cellular protein in the culture wells after washing to remove detached cells and serum. The attached cells were lysed in 0.5% Triton X-100 and assayed for protein with the bicinchoninic acid method (Smith et al., 1985). The response of control and AZT-treated cells to activation with rIFNγ was assessed by measuring H_2O_2-releasing capacity after 48 h of treatment with 100 units/ml of rIFNγ. Normal human monocyte/macrophages acquire an increased capacity to release H_2O_2 after activation with rIFNγ (Murray, 1988). Phorbol 12-myristate-13-acetate-triggered H_2O_2-release was measured with the colorimetric method of Pick and Kesari (1980) as described (Mellors et al., 1987).

Statistical analysis

The 90% inhibitory concentration (IC_{90}) of AZT was calculated from linear-$\log_{10}$ plots of % p24 antigen inhibition vs. $\log_{10}$ AZT concentration (μM). The Student two-tailed *t*-test for unpaired data was used to assess statistical significance.

Results

Anti-HIV activity of AZT in primary mononuclear phagocytes

These studies investigated whether AZT could protect normal donor peripheral blood monocytes (PBM), monocyte-derived macrophages (MDM) and alveolar macrophages (AM) from HIV-1 infection. Purified cell populations were challenged with high-titer HIV-1_{BaL} (MOI of 0.5–20) and treated with AZT (0.1 nM–10 μM) starting immediately after virus adsorption. AZT treatment was continued for the duration of each experiment (14–28 days). Fig. 1 shows the kinetics of HIV- 1_{BaL} replication in MDM from a single blood donor. HIV-1_{BaL} replicated very efficiently in no-drug control wells with supernatant p24 antigen levels exceeding 300 ng/ml by day 14 of infection. This level of p24 antigen correlated with an infectious virus titer of $>10^5$ $TCID_{50}$ per ml of supernatant indicative of highly-productive infection. Fig. 1 also demonstrates that AZT inhibited viral replication in MDM in a concentration-dependent manner (Fig. 1). Supernatant p24 antigen levels (day 14) were reduced >1.0 $\log_{10}$ by 0.01 μM AZT, >2.0 $\log_{10}$ by 0.1 μM AZT, and >3.0 $\log_{10}$ by 1.0 μM AZT. At 10 μM AZT, there was no detectable p24 antigen (<50 pg/ml) in supernatants from days 7, 10, and 14 of infection. On day 4 of infection, however, there was a low level of detectable antigen (~ 150 pg/ml). Similar amounts of p24 antigen were consistently observed in several experiments on days 4–7 of infection and may represent either transient low-level viral breakthrough or release of cell-bound viral antigens originating from the inoculum.

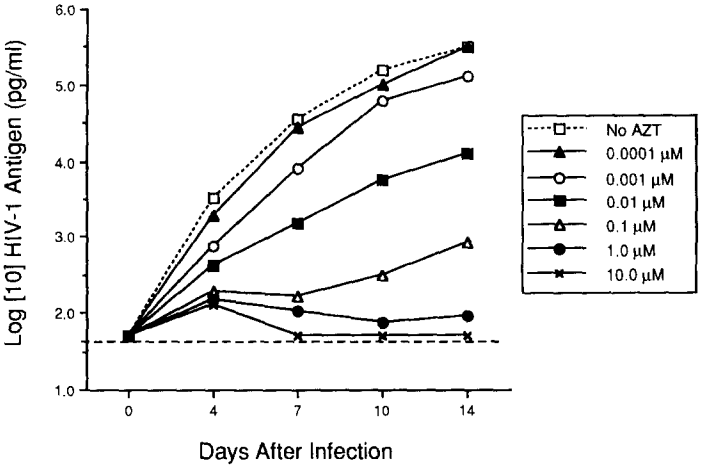

Fig. 1. Effect of AZT on the kinetics of HIV-1_{BaL} replication in monocyte-derived macrophages. The experiment shown was performed with cells derived from one donor (similar results were obtained with two other donors). The dashed line indicates the limit of detection for HIV-1 antigen (~50 pg/ml).

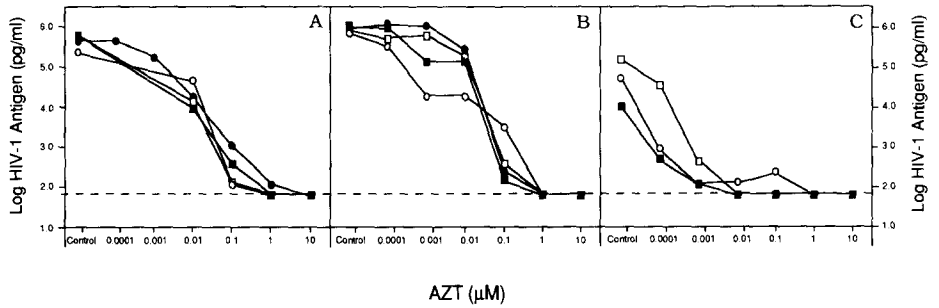

Fig. 2. Anti-HIV-1 activity of AZT in monocyte-derived macrophages (panel A), peripheral blood monocytes (panel B), and alveolar macrophages (panel C) from 3–4 different donors (each line represents a different donor). Cells were infected with HIV-$1_{Ba\text{-}L}$ and treated with the indicated concentrations of AZT for the duration of the experiment. The data shown are HIV-1 p24 antigen levels from day 14 post-infection for monocytes and monocyte-derived macrophages and from day 28 post-infection for alveolar macrophages.

Fig. 2 summarizes the antiviral activity of AZT in MDM, PBM and AM from 3–4 different donors for each cell type. The concentration–inhibition curves of AZT were very similar in a specific cell type even though cells originated from different donors. For example, HIV-1_{BaL} replication in MDM was decreased 2–3 $\log_{10}$ by 0.1 μM AZT in 4 of 4 donors and was inhibited to below the limit of detection by 1.0 μM AZT in 3 of 4 donors. The mean IC_{90} of AZT in MDM was 0.009 μM $\pm$ 0.002 (S.D.; n = 4 donors). AZT had similar antiviral activity in PBM. Viral replication was decreased >2 $\log_{10}$ by 0.1 μM AZT and to below the limit of detection by 1.0 μM AZT in 4 of 4 donors. The mean IC_{90} of AZT in PBM was 0.04 μM $\pm$ 0.03 (n = 4 donors). HIV-1_{BaL} replicated more slowly in AM compared with MDM or PBM. Supernatant p24

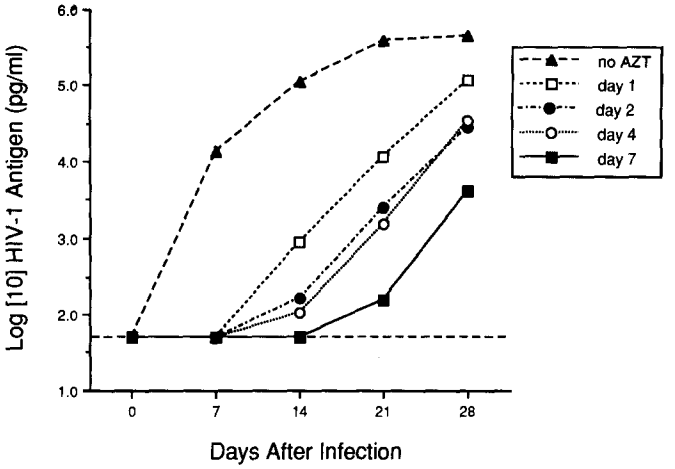

Fig. 3. Duration of anti-HIV-1 effect of AZT in monocyte-derived macrophages. AZT (1 μM) was removed from the culture medium on the days indicated, followed by several washes with PBS to remove residual AZT. Two additional experiments gave similar results.

antigen levels reached a maximum on day 28 of infection, peaking at 10–100 ng/ml (Fig. 2). AZT was remarkably potent in AM; viral replication was decreased $\geqslant 2$ $\log_{10}$ by 0.001 μM AZT in 3 of 3 donors (day 28 post-infection). The mean IC_{90} of AZT in AM was 0.1 nM $\pm$ 0.016 (n = 3 donors).

Duration of AZT inhibition

The duration of the antiviral effect of AZT in MDM was studied by removing AZT (1 μM) from the culture medium at increasing intervals after HIV-1 infection (1, 2, 4, or 7 days). Fig. 3 shows the results of a representative experiment. After stopping AZT, virus production resumed after a delay of 7–14 days and increased exponentially toward no drug-control levels. This resumption of HIV-1 replication occurred despite initial virus inhibition to below the limit of antigen detection and despite treatment of cells with AZT for as long as 7 days after infection. These experiments indicate that AZT did not permanently block infection of macrophages.

Cytotoxicity of AZT in mononuclear phagocytes

Cultured mononuclear phagocytes are fastidious cells that are readily injured or killed by in vitro treatments or manipulations. Hence, experiments were performed to carefully assess whether any of the viral inhibitory activity of AZT was attributable to direct cellular toxicity of the compound. Because macrophages are terminally-differentiated cells that are essentially non-replicating, standard methods of assessing drug cytotoxicity are not applicable (i.e., cell proliferation assays). Therefore, three parameters were used to assess drug-induced cytotoxicity: cell viability, cell mass (protein), and rIFN-γ-activated respiratory burst capacity (H_2O_2-release). The latter is a sensitive

TABLE 1

Cytotoxicity of AZT in primary mononuclear phagocytes: cell number, cell protein, and interferon-γ activated H_2O_2-release

Cell type[a]	Concentration of AZT			
	no AZT	0.1 μM	1 μM	10 μM
	Cell Number (10^3/cm^2)[b]			
PBM	4.8 ± 1.3	5.6 ± 1.8	3.7 ± 0.9	4.3 ± 0.8
MDM	3.5 ± 1.1	2.5 ± 0.6	4.6 ± 0.9	3.6 ± 0.6
AM	2.1 ± 0.5	2.1 ± 0.8	1.8 ± 0.3	1.6 ± 0.4
	Cell Protein (μg/cm^2)[b]			
PBM	113 ± 10	124 ± 32	101 ± 57	136 ± 24
MDM	151 ± 16	122 ± 11	139 ± 42	135 ± 23
AM	82 ± 10	99 ± 7	70 ± 16	62 ± 12
	H_2O_2-release (nmols/90 min)[c]			
PBM	19.6 ± 3.3	17.0 ± 3.9	22.3 ± 2.3	18.3 ± 5.1
MDM	24.3 ± 1.2	19.2 ± 5.8	17.9 ± 2.8	22.0 ± 4.0
AM	9.3 ± 1.5	9.4 ± 2.1	11.4 ± 1.4	7.9 ± 1.9

[a]PBM, peripheral blood monocytes; MDM, monocyte-derived macrophages; AM, alveolar macrophages. [b]Results are expressed per cm^2 of surface area to normalize for differences in well sizes. [c]Cells were incubated with 100 units/ml of recombinant human interferon-γ for 48 h prior to determining PMA-triggered H_2O_2-release. The data are expressed as nanomoles of H_2O_2 released in 90 min per μg of cell protein to normalize for differences in cell mass. Control cells not treated with interferon-γ released < 1.0 nmol of H_2O_2 in 90 min per μg protein. [b,c]Cultures were prepared in triplicate under each condition. The data shown are the mean values ± S.E.M. from two experiments.

measure of the ability of mononuclear phagocytes to acquire specialized microbicidal functions.

Cytotoxicity was assessed in uninfected MDM and PBM after 14 days of AZT treatment and in AM after 28 days of AZT treatment. The results of cytotoxicity assays are summarized in Table 1. In PBM and MDM, AZT was not cytotoxic at $\leqslant 10$ μM for all parameters studied. In AM, AZT was not cytotoxic at $\leqslant 1$ μM, but 10 μM AZT produced a mean 22% decrease in viable cell number, 25% decrease in cell protein, and a 15% decrease in rIFNγ-activated H_2O_2-release. These trends were not statistically significant ($P > 0.05$).

Discussion

The results presented here demonstrate that AZT is a potent inhibitor of HIV-1 replication in several types of primary human mononuclear phagocytes derived from many different donors. Our studies were performed in purified cell populations challenged with a defined viral inoculum that consistently

achieved highly-productive infection of untreated control cells. Under these experimental conditions, IC_{90} values of AZT were 0.04 μM in blood monocytes and 0.009 μM in monocyte-derived macrophages. The IC_{90} of 0.04 μM in PBM is very similar to the finding by Perno et al. (1988) that 0.05 μM AZT decreased HIV-1 replication by >95% in fresh elutriated monocytes. These investigators reported that two-fold higher AZT concentrations (0.1 μM) were required to achieve 95% inhibition of HIV-1 in 5-day cultured monocyte/macrophages. Our studies with 14-day cultured macrophages indicate that these fully-differentiated cells are somewhat more sensitive (~two-fold) than fresh monocytes to the protective effects of AZT. This minor difference between our results and those of Perno et al. (1998) is probably attributable to differences in the methods of cell purification and the duration of culture before HIV-1 infection (5 days vs. 14 days). It is not clear, however, why our results and those of Perno and others (Skinner et al., 1988; Szebeni et al., 1989) differ from the initial report by Richman et al. (1987) that AZT and other ddNs did not inhibit HIV-1 in monocyte-derived macrophages at concentrations as high as 100 μM. In the studies by Richman et al. (1987), the lymphotropic LAV-1 strain was used to infect macrophages and p24 antigen production was assessed at an early time point after infection (3 days). Perhaps at this early time point a significant portion of the p24 antigen in the culture supernatants was residual antigen from the viral inoculum. Kinetic analyses of HIV-1 replication in macrophages (Fig. 1 and Perno et al., 1988) indicate that de novo viral antigen production is minimal three days after infection even with highly-productive macrophage-tropic strains.

Our work extends the findings described above to a pure population of tissue macrophages harvested from an organ (lung) that is the site of many opportunistic infections in patients with HIV-1 infection. Recently, Hammer et al. (1990) reported that high concentrations of AZT (1 μM) can inhibit the low-level productive infection of alveolar macrophages achieved with the lymphotropic HIV-1_{IIIB} strain. Our studies support the initial findings of Hammer et al. (1990) and provide additional evidence that AZT is a potent inhibitor of highly-productive HIV-1_{BaL} infection of alveolar macrophages. Our comparative studies also demonstrate that alveolar macrophages are more sensitive to the protective effect of AZT than are blood monocytes or in vitro-derived macrophages.

A somewhat unexpected finding was that HIV-1 replication in macrophages resumed after stopping AZT despite initial viral inhibition to below the limit of p24 antigen detection. Hammer et al. (1990) have observed a similar phenomenon in alveolar macrophages. Alveolar macrophages that had been treated with AZT for 20 days after HIV-1 challenge could still infect PHA-stimulated T-cells if AZT was removed from the medium after addition of the fresh target cells. In contrast to these findings, Mitsuya et al. (1987) reported that continuous treatment of the ATH8 T-cell line with dideoxycytidine (ddC) for 30 days following viral challenge was sufficient to prevent relapse of infection after drug removal. However, ATH8 cells continuously proliferate

and thus a small subpopulation of infected cells may have been eliminated by serial passage of the cell line over the 30-day interval of ddC treatment.

There are at least two potential explanations for the observed resumption of HIV-1 replication in macrophages after removal of AZT. First, inhibition of HIV-1 reverse transcriptase (RT) by AZT-5′ triphosphate (AZTTP) may be a reversible event. AZT-TTP is believed to inhibit retroviral reverse transcription by two mechanisms: (1) competitive inhibition of normal substrate (dNTP) binding to RT; and (2) incorporation of AZT-monophosphate in retroviral DNA resulting in chain termination (Mitsuya et al., 1985; Mitsuya and Broder, 1986; Furman et al., 1986). The relative importance of these two mechanisms is not known. Competitive inhibition of normal substrate binding is likely to be a reversible process, whereas DNA chain termination would not be reversible in the absence of DNA repair (i.e., excision of AZT-monophosphate). The absence of known proof reading or repair function of HIV-1 RT makes chain termination likely to be permanent. The potential reversibility of RT inhibition by AZT-TTP thus depends on which mechanism of AZT action predominates intracellularly (competitive inhibition vs. chain termination).

An alternative explanation for the resumption of HIV-1 replication after AZT removal is that a minor subpopulation of macrophages is not initially protected by AZT (e.g., because of inadequate cellular activation of AZT to AZTTP). Once AZT is removed, virus produced by this infected subpopulation could then infect other macrophages, leading to spread of infection throughout the culture. An initial subpopulation of infected cells would not be detected if the amount of virus produced by these cells was below the sensitivity of the p24 antigen assay ($\sim$50 pg/ml). More sensitive methods of virus detection such as the polymerase chain reaction may help resolve this issue.

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