

INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS
(HIV-1/HTLV-III_{Ba-L}) REPLICATION IN FRESH AND
CULTURED HUMAN PERIPHERAL BLOOD
MONOCYTES/MACROPHAGES BY AZIDOTHYMIDINE AND
RELATED 2',3'-DIDEOXYNUCLEOSIDES

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The infection and destruction of T lymphocytes by human immunodeficiency virus (HIV) has been considered a central event in the pathogenesis of the acquired immunodeficiency syndrome (AIDS) (1-3). There is a growing body of evidence, however, that infection of cells in the monocyte/macrophage (M/M)¹ series by HIV may be clinically as important as that of T cells (4-7). Certain strains of HIV can replicate in M/M for a considerable time (>6 mo) without necessarily inducing a substantial cytopathic effect (4, 8). Such cells may thus serve as a reservoir for dissemination of the virus to other cell types. Furthermore, HIV infection can perturb accessory cell function without killing cells (9 and Mitsuya et al., unpublished data). In addition, M/M are believed to be important target cells in the pathogenesis of AIDS-related dementias (10-12).

Our group has recently reported that several members of a family of compounds known as dideoxynucleosides (ddN) are potent inhibitors of HIV replication in human T cells (13, 14) *in vitro*, and at least two such compounds, 3'-azido-2',3'-dideoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC) have been shown to have antiretroviral activity in patients with AIDS and AIDS-related complex (ARC) (15-17). Several of these drugs are currently being studied in clinical trials.

ddN are thought to require anabolic phosphorylation to an active triphosphate moiety (ddNTP) by kinases (dN kinase) in the relevant target cells (18-21). As triphosphates, these compounds act at the level of the viral reverse transcriptase (RT),

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¹ *Abbreviations used in this paper:* ARC, AIDS-related complex; AZT, 3'-azido-2',3'-dideoxythymidine; ddN, 2',3'-dideoxynucleosides; ddNDPch, 2',3'-dideoxynucleoside-5'-diphosphatidylcholine; ddNTP, 2',3'-dideoxynucleoside-5'-triphosphate; dN, 2'-deoxynucleosides; dNTP, 2'-deoxynucleoside-5'-triphosphate; DTT, dithiothreitol; HTLV, human T leukemia/lymphoma (lymphotrophic) virus; LAV, lymphadenopathy-associated virus; M/M, monocyte/macrophages; RT, reverse transcriptase.

competing with the normal nucleoside triphosphates (dNTP) (18, 22, 23), and possibly terminating proviral DNA chain elongation. These drugs can prevent uninfected cells from becoming so, but they do not eliminate virus from chronically infected cells (Mitsuya et al., unpublished data).

It is known that M/M may have lower levels of nucleoside kinase activities compared with replicating T cells (24), although the absolute levels of these enzymes may be affected by the status of differentiation of M/M (25-28). It has also been reported that certain members of the ddN family may be phosphorylated more slowly in M/M than in T cells (24). Because of these differences and the importance of M/M in HIV infection, we examined the ability of three ddN that are currently being evaluated in clinical trials to inhibit HIV replication in M/M, and correlated the activity of two of them with the concentration of their triphosphorylated moiety in such cells.

In this study we have learned that each of these ddN tested can suppress the *in vitro* replication of a monocytoprotic strain of HIV, HTLV-III_{Ba-L}, in M/M at concentrations comparable to those at which T cells are protected. We further show that, although the phosphorylation of at least one of these compounds, AZT, is substantially reduced in M/M as compared with T cells, there is also a parallel reduction in the levels of the competing normal dNTP. Taken together, the results suggest that the ratio of the ddNTP to the normal dNTP may be a crucial factor in determining the activity of ddN against HIV.

Materials and Methods

Drugs. AZT (Wellcome Research Laboratories, Research Triangle Park, NC), 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxyadenosine (ddA) (Pharmacia Fine Chemicals, Piscataway, NY) were diluted in sterile distilled water or in RPMI 1640 and kept at 4°C until used. [³H]ddC (sp act 27 Ci/mmol) (Research Triangle Institute, Research Triangle Park, NC) and [³H]AZT (sp act 3 Ci/mmol) (Moravek Biochemical, Brea, CA) were stored dried and used immediately after reconstitution.

Virus. Two different strains of HIV-1, HTLV-III_{Ba-L/85} and HTLV-III_{B/83}, were used in these experiments. HTLV-III_{Ba-L}, which was originally obtained from a sample of lung tissue, and which replicates efficiently in monocytes (4), was maintained in cultures of normal peripheral blood monocytes. HTLV-III_B (1), which efficiently replicates in cultured T cells, was obtained from the concentrated supernatant of infected H9 cells (Electro-Nucleonics Laboratory, Inc., Silver Spring, MD). Different strains of HIV-1 were used to infect T cells and M/M populations because we obtained only inconsistent infection of M/M using HTLV-III_B, and similarly inconsistent infection of immortalized T cell lines using HTLV-III_{Ba-L}. The viral preparations were stored in liquid nitrogen. All viral preparations were tested and found to be free of contamination with mycoplasma.

Cells. Monocyte-enriched populations of peripheral blood leukocytes were obtained from healthy, HIV⁻ donors, using a Fenwal C3000 cell separator. These cells were further enriched for M/M by centrifugation over Ficoll-Hypaque (Organon Teknika Co., Durham, NC). An additional purification of M/M was done using three different methods. (a) Fresh M/M were obtained by elutriation using a Beckman J-6B centrifuge (Beckman Instruments, Palo Alto, CA) as previously described by Gerrard et al. (29). (b) Fresh M/M were also obtained by 2-h adherence to 75-cm² plastic flasks (Costar, Cambridge, MA), in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 20% heat-inactivated FCS (Gibco), 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (Gibco), referred to as complete medium. The nonadherent cells were then removed by three washings with warm RPMI 1640 medium, and the adherent cells were detached by a 15-min incubation at 37°C with 10 ml Versene, 1:5,000 (Gibco) at 37°C. (c) 5-d-old adherent cells were obtained by incubating

10^6 PBMC/well in 48-well (1-cm² well) plates (Costar) in 1 ml of complete medium. Nonadherent cells were removed by washing the wells several times with RPMI 1640 at 37°C, and the adherent cells were left attached. In general 0.8×10^5 – 1.5×10^5 viable cells remained attached to each well, as detected by trypan blue exclusion.

Viability of the cells obtained by each of these techniques was always >95%, as determined by trypan blue exclusion. In each population, ≥95% of the cells had the morphologic characteristics of M/M when examined after Giemsa staining (Gibco). 5-d adherent cells were consistently >97% positive for nonspecific esterase (Technicon Instruments Co., Tarrytown, NY), while the elutriated and 2-h adherent cells were >80% esterase positive when initially prepared; these latter two preparations became >95% esterase positive after being cultured for 5 d in complete medium. The 5-d adherent cells were >75% positive for OKM1 (Ortho Diagnostic Systems, Inc., Raritan, NJ), while the elutriated and 2-h adherent cells were consistently >95% positive for OKM1. Cells prepared by each of the techniques were <1% E-rosette positive (30) and <1% OKT11⁺ (Ortho Diagnostic Systems, Inc.). Two cell lines were used in certain experiments: ATH8, and HTLV-1-infected T4⁺ cell clone that is highly sensitive to the cytopathic effect of HIV-1 (HTLV-III_B strain) (13), and H9, a T4⁺ cell line (1); these lines were both mycoplasma free. In some experiments, macrophage-depleted peripheral blood lymphocytes were used; they were consistently <2% positive by nonspecific esterase staining.

Assays of Antiviral Activity. Immediately after separation, 10^6 fresh M/M (prepared by either elutriation of 2-h adherence) were seeded in 24-well plates (2 cm²/well) (Costar) in 2 ml of complete medium. In some experiments, 1- or 4-cm² well plates were used, keeping the same cell concentration: no difference was seen in the results. Also, in a few experiments, 10^5 fresh elutriated M/M were cultivated in 1 ml of complete medium in 1-cm² well plates for 5 d before drug exposure and challenge with HTLV-III_{Ba-L}. 5-d adherent M/M were allowed to remain attached in the 1-cm² wells in 1 ml of complete medium after the nonadherent cells were washed off on day 5. Neither IL-2 nor mitogens were added to any of the M/M cultures. In preliminary experiments, we found that preincubation of M/M with polybrene did not improve infection of the cells (data not shown); thus polybrene was omitted in these experiments.

Before exposure to HIV, the target cells were preexposed to various concentrations of AZT, ddC, or ddA for 20 min; they were then exposed to HIV without washing out the drugs. Unless stated otherwise, 80,000 cpm/ml RT of HTLV-III_{Ba-L} were used in the experiments; this represented 0.16 RT cpm/cell for the elutriated and 2-h adherent cells, and 0.5–1 RT cpm/cell for the 5-d adherent cells (depending on the number of cells remaining attached to the wells). This dose of virus generally yielded the maximum infection of the M/M populations, and was 16–100 times the minimum infective dose (depending on the number of cells present in the wells). In some experiments, 3,000 viral particles/cell of HTLV-III_B were used to infect ATH8 or H9 T cell lines; this is 60 times the minimum infective dose for ATH8 cells. Appropriate positive and mock-infected negative controls were run for each experiment.

After infection, the cells were maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂. 2 d after infection, M/M were extensively washed to remove excess virus, and cultivated in 2 ml of complete medium with the same concentration of drugs as before. Cells were washed and fed every 4 d.

To evaluate any initial virus remaining after washing, in each experiment 1 ml of complete medium plus 80,000 RT cpm/ml of HTLV-III_{Ba-L} was added to each of two wells in the absence of cells. The wells were washed and fed at day 2, and subsequently every 4 d, comparable to the procedure for cell-containing wells. The initially added virus was considered absent when both wells contained undetectable HIV-p24 antigen (see below).

Viral Detection. Starting from day 3, HIV production was assessed regularly until day 28 using two different methods: measurement of released RT activity, and detection of HIV-p24 antigen production in culture supernatant. To measure RT, 1 ml of cell-free supernatant was precipitated with 0.5 ml polyethylene glycol (mol wt 8,000) (Sigma Chemical Co.) and the RT was assayed using a minor modification of a technique previously described (31). Briefly, 10 μl of protein extract was incubated for 1 h at 37°C with 50 mM tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 20 μM [³H]dTTP (New England Nuclear, Boston, MA) and 5

μg of poly(rA)-oligo(dT)₁₂₋₁₈ (Pharmacia Fine Chemicals) in a final volume of 100 μl . The reaction was stopped with 10 μg of yeast transfer RNA plus 1 ml of chilled 10% TCA containing 0.02% sodium pyrophosphate. The precipitates were collected on glass fiber filters, washed and dried; after addition of 10 ml of nonaqueous liquid scintillation cocktail (New England Nuclear) the radioactivity was determined with a Beckman Model scintillation spectrometer. HIV-p24 antigen was assessed by a sandwich ELISA (Dupont Co., Wilmington, DE). With these two methods, a small amount of residual virus (50–100 pg/ml of p24) was sometimes present at day 3. However, residual virus was never detected at day 7 or later.

Additional experiments were performed using unstimulated peripheral blood lymphocytes, H9 cells, and ATH8 cells. H9 cells were cultivated in complete medium without IL-2. ATH8 cells were cultured in complete medium supplemented with 15% human natural IL-2 (ABI, Silver Spring, MD) and 20 U/ml human recombinant IL-2 (AMGen Biological, Thousand Oaks, CA). In the experiment with ATH8 as a target of HIV-infection, protection from the cytopathic effect of HIV/HTLV-III_B (a measure of antiretroviral effect) was assessed by trypan blue exclusion as previously described (32). It is worth stressing, however, that one must be cautious in comparing the activity of drugs in the T cell and M/M assay systems used in this work, because different strains of HIV were, of necessity, used in the two cell types. Drug toxicity was evaluated at various times by trypan blue exclusion in mock-infected, drug-exposed M/M.

Metabolism of ddN. 5×10^6 fresh elutriated M/M and 2-h adherent M/M (immediately after separation), 5-d-old M/M (immediately after final purification), and H9 cells were plated in 2 ml of complete medium in 2-cm² well (Costar), and treated for 24 h with 0.5 μM undiluted, [³H]ddC, or with 10 μM undiluted, [³H]AZT. The cells were then harvested (M/M by gentle scraping followed by washing with cold RPMI), counted (viability was always >92%), and washed three times in cold RPMI to remove excess radioactivity, pelleted, and immediately frozen in dry ice. Cell pellets were then extracted with 0.5 ml 10% TCA; the acid extract was neutralized with an equal volume of tri-*n*-octylamine in freon, after which the neutralized extracts were analyzed via HPLC for metabolites (19, 20). Results were given as picomoles of ddCTP or AZTTP per 10^6 cells.

dNTP Pools. 5×10^7 elutriated, 2-h adherent, or 5-d adherent M/M were collected immediately after preparation (see above), washed at 4°C with RPMI, pelleted and stored at -70°C; a similar procedure was followed for H9 cells. Endogenous dNTP pools were analyzed by anion exchange HPLC. Pellets were extracted with 10% TCA and neutralized as shown in the previous paragraph. Ribonucleotides were degraded using a modification of the periodate procedure of Garrett and Santi (33). The HPLC system used a Whatman Partisphere 5- μm SAX column (Whatman International Ltd., Maidstone, England) and a gradient of 0.01–0.6 M ammonium phosphate, pH 3.5 (19, 20).

Nucleoside Kinase Assay. 10^7 fresh elutriated M/M, 5-d-old M/M, and H9 cells prepared as described above were washed in cold RPMI; the pellet was resuspended in 200 μl of 2 mM dithiothreitol (DTT) and stored at -70°C. 2'-deoxycytidine kinase (dC kinase) and 2'-deoxythymidine kinase (dT kinase) activities were measured by a minor modification of a published procedure (34) using 10 or 2,000 μM 2'-deoxycytidine (dC) or 430 μM dT as substrate in the presence or absence of 500 μM ATP-Mg.

Results

Time Course of Infection of M/M by HTLV-III_{Ba-L}. The time course of HIV production by fresh elutriated and 5-d adherent M/M following exposure to HTLV-III_{Ba-L} is shown in Fig. 1. No detectable viral production was found during the first 3 d after viral exposure. However, starting at about 10 d, high-titer viral production was obtained which was sustained for at least 4 wk. In most experiments we observed greater virus production in 5-d adherent M/M than in fresh (elutriated or 2-h adherent) cells (Fig. 1).

The efficiency of infection was dependent on the concentration of the added virus (Table I). Peak infection was obtained with 80,000 RT cpm/ml of culture. When

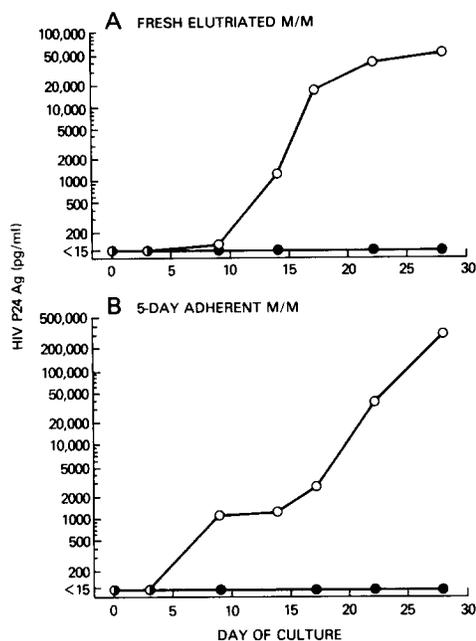


FIGURE 1. Kinetics of HTLV-III_{Ba-L} infection in fresh elutriated M/M and 5-d-old M/M with and without 0.1 μ M ddC. (A) Fresh elutriated M/M were exposed to 80,000 cpm RT/ml of HTLV-III_{Ba-L} and cultured in complete medium as described. The p24 antigen in the supernatant was assayed at different times. \circ , Cultures with media alone; \bullet , cultures with 0.1 μ M ddC. (B) Same as in A, except that 5-d adherent M/M were used.

higher concentrations of virus were added to the cultures, the viral production was often lower, possibly because of early interference by incomplete viral particles. It is also conceivable that a cytopathic effect of the viral preparation may have contributed to this; however, little or no cell death was observed when HTLV-III_{Ba-L}-exposed M/M were assessed by trypan blue exclusion.

Little or no viral production was found in cultures of monocyte-depleted PBL exposed to HTLV-III_{Ba-L} in the absence of PHA and IL-2 (results not shown).

Anti-HIV Activity of ddN in M/M. Based on these initial experiments, we examined the anti-HIV effect of several ddN in cultures of M/M exposed to 80,000 RT cpm/ml of HTLV-III_{Ba-L}. In cultures of fresh M/M (obtained either by elutriation or by 2-h adherence), we found $\geq 95\%$ suppression of HIV replication with concentrations of 0.05 μ M AZT, 0.01 μ M ddC, or 0.05 μ M ddA (Figs. 1 and 2). These concentrations of drugs are lower than those required to suppress the virus in H9, a replicating T cell line, against infection with the T cell-tropic virus HIV/HTLV-

TABLE I
Viral Production in Fresh Elutriated M/M Exposed to Different Doses of HIV/HTLV-III_{Ba-L}

HIV added to the cultures at day 0	HIV p24 antigen in M/M culture supernatants		
	Day 14	Day 21	Day 28
<i>cpm RT/ml</i>		<i>pg/ml</i>	
20,000	<15	722	7,165
80,000	345	65,368	52,453
320,000	49	23,097	46,460

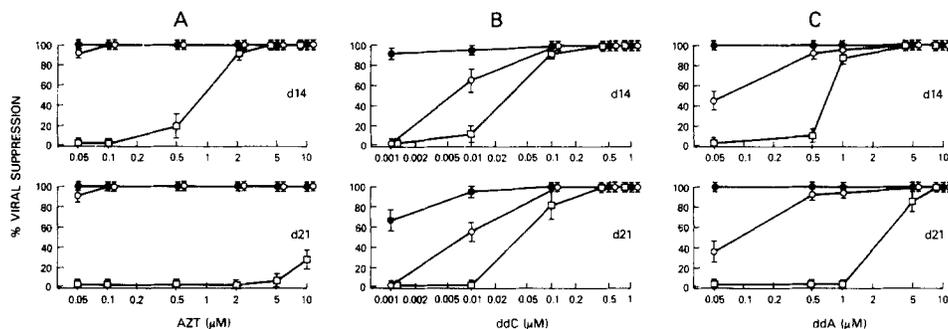


FIGURE 2. Viral suppression as a function of drug concentration in M/M and H9 cells exposed to HIV-1 in presence of various concentrations of ddN. (A) Protection by AZT as assessed by HIV p24 antigen at day 14 (*top*) and day 21 (*bottom*). The cells tested are as follows: ●, fresh elutriated M/M; ○, 5-d-old adherent M/M; □, H9 cells. HTLV-III_{Ba-L} was used to infect the monocytes and HTLV-III_B was used to infect the H9 cells. Control p24 antigen production (without drugs) is as follows: Fresh elutriated M/M, 1,300 pg/ml at day 14 and 48,500 pg/ml at day 21; 5-d M/M, 36,000 pg/ml at day 14 and 320,700 pg/ml at day 21; H9 cells, 105,000 pg/ml at day 14 and 98,000 pg/ml at day 21. (B) Protection by ddC, expressed in a similar manner. (C) Protection by ddA, expressed in a similar manner.

III_B (Fig. 2). Protection was sustained throughout the 28-d culture period (Fig. 1 *a*). In additional experiments, no viral production was detected when either fresh or 5-d adherent M/M were exposed up to 320,000 cpm/ml RT of HTLV-III_{Ba-L} in the continuous presence of 0.5 μ M ddC (data not shown).

Each of the ddN tested could also suppress HTLV-III_{Ba-L} replication in 5-d adherent M/M, although in this cell population somewhat higher concentrations of drugs were required. In most experiments, we observed >95% inhibition of viral replication in 5-d-old M/M with 0.1 μ M AZT, 0.1 μ M ddC, or 1 μ M ddA (Fig. 2). Similar results were obtained when less virus (20,000 cpm RT/ml) was used to infect the cells. Again, viral inhibition was sustained for each drug during the whole 28-d period assayed. In additional experiments, we examined the effect of ddN on HIV infection of fresh elutriated M/M that were allowed to mature for 5 d in culture before being exposed to virus (Table II). AZT was slightly less potent in this cell population than in either fresh or 5-d adherent cells; the ID₅₀ was \sim 0.1 μ M. The activities of ddC and ddA were roughly similar in these cells to their activity in 5-d adherent cells (ID₅₀ \sim 0.001 μ M for ddC and 0.1–1 μ M for ddA).

In some experiments, elutriated or 5-d-old M/M were subjected to three cycles of freezing and thawing at day 21, with the aim of releasing viral antigens that might be in the macrophages; this technique has been shown to be effective at releasing viral antigens in other cell culture systems (35) and has been recently reported by Gendelman et al. (36) to induce the release of RT from HIV-infected macrophages cultured with colony-stimulating factor. No additional p24 antigen was detected in the supernatants after this procedure (data not shown). Also, in three different experiments, HIV production was assayed by a determination of RT activity in culture supernatants; these results were essentially parallel to those assessed by p24 antigen production (data not shown). Finally, M/M exposed to high multiplicity of infection of HIV-1 in the form of HTLV-III_B (a viral isolate that can readily replicate within T cells) showed inconstant low-level infection; however the viral production that did occur was consistently inhibited by ddN (data not shown).

TABLE II
*Suppression of HTLV-III_{Ba-L} Replication by AZT, ddC, and ddA
 in Elutriated M/M Cultivated for 5 d before Viral Exposure*

Drug	Concentration μM	HIV-p24 antigen level pg/ml	Viral suppression %
No drug	—	57,200	0
AZT	0.01	42,304	26
	0.1	33,017	43
	1	4,162	93
	10	<31	100
ddC	0.001	34,175	40
	0.01	<31	100
	0.1	<31	100
ddA	0.01	66,428	0
	0.1	49,824	13
	1	<31	100

10^5 elutriated M/M were cultivated for 5 d in 1 ml of complete medium. They were then exposed to AZT, ddC, or ddA for 20 min and challenged with HTLV-III_{Ba-L} 80,000 RT cpm/ml (0.8 RT cpm/cell) while maintaining the drugs in the media. Viral replication was assessed at day 21 by HIV-p24 antigen production.

Consistent with the monocytotropic nature of HTLV-III_{Ba-L}, ATH8 cells (an immortalized T cell clone) exposed to HTLV-III_{Ba-L} at 80,000 RT cpm/ml failed to produce detectable virus up to day 14 after viral exposure, while only transient and low-level viral production was observed in H9 T cells exposed to this strain. Using HTLV-III_B, >95% protection at day 14 was obtained in both T cell lines with 2 μM AZT, 0.5 μM ddC, or 5 μM ddA. These concentrations of ddN (Fig. 2 and data not shown) are somewhat higher than those required to protect M/M; however, one must use caution in making stringent comparison between M/M and H9, because different strains of virus were used in different cells. At day 21 there was a viral breakthrough in H9 cells with AZT, even at the highest dose tested (10 μM) (Fig. 2 A), in agreement with previously reported results (37, 38).

No toxicity (as assessed by trypan blue exclusion) was observed in M/M (prepared by any of the three methods) exposed for 14 d to up to 20 μM AZT, 2 μM ddC, and 100 μM ddA (Fig. 3); thus the doses of ddN that induced viral inhibition in M/M are much lower (at least 20–200 times) than those necessary to kill the target

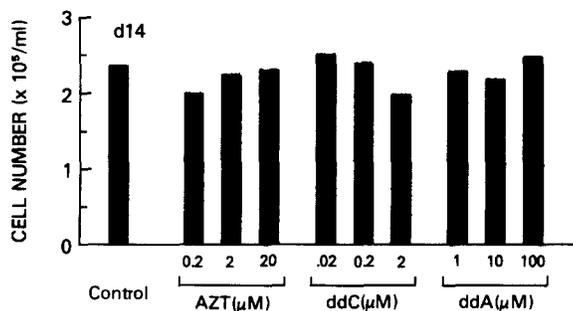


FIGURE 3. Toxicity of AZT, ddC, and ddA in elutriated M/M. M/M were cultivated in 2 ml of complete medium in 2-cm² wells, in presence of various concentrations of AZT, ddC, and ddA. After 14 d, M/M were detached by versene plus scraping, and were counted in a hemocytometer chamber; viability was evaluated by trypan blue exclusion. As additional control, the same experiment was run in polypropylene tubes using the same experimental conditions: no difference was seen in the results.

cells. Therefore, it is unlikely that the antiviral effect of ddN is an artifact of drug toxicity. Thus, although there were some differences between fresh and 5-d adherent M/M, both populations could be protected against HIV infection with each of the ddN congeners tested.

To better define the basis of this antiviral activity, we evaluated the anabolic phosphorylation of AZT and ddC in M/M and compared it with the phosphorylation in H9 T cells. In addition, we examined the pool size of the endogenous normal dNTP, 2'-deoxythymidine-5'-triphosphate (dTTP), and 2'-deoxycytidine-5'-triphosphate (dCTP) in each of these cell populations. As seen in Table III, similar levels of ddCTP were produced in fresh-elutriated M/M and H9 cells exposed to 0.5 μM of [^3H]ddC for 24 h, while 5-d adherent M/M cells accumulated slightly lower levels of ddCTP. A typical chromatographic elution pattern of ddC and its phosphorylated metabolites in elutriated M/M and H9 cells is illustrated in Fig. 4 A. In addition, fresh elutriated M/M and H9 cells had comparable activity of dC kinase, the enzyme that catalyzes the first phosphorylation of ddC (Table IV); 5-d adherent M/M expressed somewhat lower dC kinase activity. Since ddCTP is thought to compete with the normal dCTP at the level of viral reverse transcriptase, we further examined the level of dCTP in these cell populations. As can be seen in Table III, the levels of dCTP in either M/M population were <20% of that in H9 cells. Thus, the protective effect of ddC in M/M, in spite of somewhat reduced ddCTP formation, may have been produced by a substantially lower competing dCTP pool size in these cells.

When AZT metabolism was examined in these cells, a somewhat different pattern was seen. Both fresh elutriated and 5-d adherent M/M exposed to 10 μM [^3H]AZT for 24 h had substantially reduced levels of AZTTP as compared with H9 T cells (Table III). A typical chromatogram is shown in Fig. 4 B. Also the levels of dT kinase activity were markedly reduced in the M/M cells as compared with T cells (Table IV). However, when dTTP was assayed, both M/M populations had <5% of the concentration of dTTP found in H9 T cells (Table III). Thus, although AZT is phosphorylated less efficiently in M/M than in H9 T cells, there are substantially reduced levels of the competing dTTP in M/M; as a result the ratio of AZTTP to dTTP is roughly similar in M/M to that in H9 T cells.

TABLE III
Phosphorylation of ddC and AZT to the 5'-Triphosphate, and Levels of Corresponding Normal Nucleoside Triphosphates in Monocyte/macrophages and H9 T Cells

	ddCTP*	dCTP [†]	AZTTP [§]	dTTP [‡]
	<i>pmole/10⁶ cells</i>			
Elutriated M/M	0.82 \pm 0.19	1.95	0.034 \pm 0.018	0.58
5-day-old M/M	0.64 \pm 0.36	2.45	0.082 \pm 0.033	0.76
H9 cells	0.93 \pm 0.37	15.4	0.31 \pm 0.12	21.45

Results shown for ddCTP and AZTTP are the mean \pm SD of three to six experiments. For dCTP and dTTP, the results are the average of two experiments.

* ddCTP in cells exposed to 0.5 μM [^3H]ddC for 24 h.

[†] dCTP or dTTP in cells not exposed to drugs.

[§] AZTTP in cells exposed to 10 μM [^3H]AZT for 24 h.

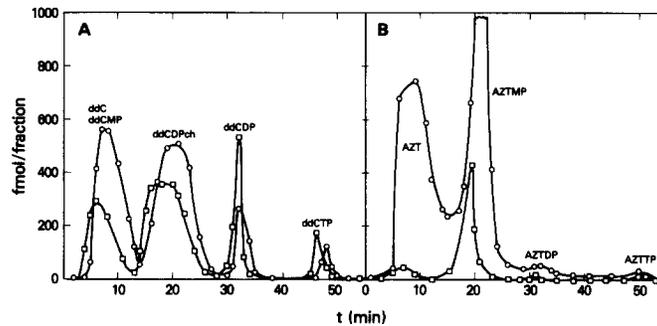


FIGURE 4. Typical ion-exchange (Partisil-10 SAX) HPLC elution profile of 10% TCA extracts of H9 T cells and fresh elutriated M/M incubated for 24 h with $0.5 \mu\text{M}$ [^3H]ddC (A) or $10 \mu\text{M}$ [^3H]AZT (B). Analyses were carried out using radial compression columns of Partisil-10 SAX equilibrated and developed with 0.01 M ammonium phosphate, pH 3.6, for 15 min followed by a linear gradient to 0.6 M ammonium phosphate, pH 3.8, over the next 25 min, and finally by a 5 min isocratic elution with 0.6 M ammonium phosphate, pH 3.8. TCA extracts were neutralized with tri-*n*-octylamine in freon before analyses. Sample volume: $200 \mu\text{l}$. 1-min fractions were collected and data are expressed as femtomoles per fraction per 10^6 cells. The AZTMP peak in B for the H9 cells extract is off-scale for the 20-, 21-, and 22-min fractions: the AZTMP values for these fractions were 3,888, 7,557, and 2,524 fmoles/fraction/ 10^6 cells, respectively. \square , Elutriated M/M; \circ , H9 T cells.

The ddA metabolism was not evaluated because of the technical difficulty in accurately measuring the final product of ddA anabolism, 2',3'-dideoxyadenosine-5'-triphosphate (ddATP). However the concentrations of endogenous 2'-deoxyadenosine-5'-triphosphate (dATP) in both fresh and 5-d adherent M/M was $<0.5 \text{ pmole}/10^6$ cells, compared with $17.35 \text{ pmole}/10^6$ cells in H9 cells. Thus, in the case of ddA as well, a small pool size of the competing dNTP in M/M may permit anti-HIV activity of ddA even if its level of phosphorylation is low.

Discussion

It has become increasingly apparent that infection of cells of the M/M lineage by HIV plays an important role in the pathogenesis of AIDS (4, 39). As these cells may be resistant to the cytopathic effect of HIV, once infected, they could persistently produce HIV (4, 7, 8). Because of this, they could serve as a reservoir of HIV and in this context frustrate certain antiretroviral chemotherapies. In addition, since HIV envelope-induced syncytia formation can lead to T cell death without the need

TABLE IV
2'-Deoxycytidine Kinase and 2'-Deoxythymidine Kinase in Fresh Elutriated M/M, 5-d-old M/M, and H9 T Cells

	2'-Deoxycytidine kinase <i>pmole dCMP/mg protein/min</i>	2'-Deoxythymidine kinase <i>pmole dTMP/mg protein/min</i>
Elutriated M/M	9.44 ± 2.33	34.2 ± 14.05
5-d-old M/M	2.62 ± 1.59	5.7 ± 1.11
H9 cells	9.07 ± 2.52	407.7 ± 161.5

Results shown are the mean \pm SD of three experiments. dCMP, 2'-deoxycytidine-5'-monophosphate; dTMP, 2'-deoxythymidine-5'-monophosphate.

for such T cells to become infected per se, HIV-producing macrophages could theoretically cause T cell depletion even if the T cells are rendered virally resistant by drugs (40, 41). Finally, M/M are an important target cell for HIV in the brain (10, 11) and thus may play a role in the pathogenesis of HIV-induced dementia (12, 39). For these reasons, it is crucial that an antiviral strategy for HIV infection somehow address the infection of M/M by the virus.

Mature M/M have a limited proliferative potential, and certain studies of these cell populations (42-44) have suggested that they may phosphorylate nucleosides poorly as compared with dividing lymphocytes. In agreement with previous studies of others (24), we found in these experiments that the phosphorylation (metabolic activation) of at least one ddN analogue, AZT, was substantially reduced in monocytes; however, the metabolic activation of another dideoxynucleoside, ddC, was roughly comparable to that in T cells. It was therefore somewhat surprising that HIV replication could be suppressed in M/M by each ddN tested (including AZT) at concentrations comparable to those at which the drugs inhibit HIV in T cells. To further explore this apparent incongruity, we examined the level of dTTP and dCTP in the M/M populations, and found that the level of dCTP was six to eight times lower than that in T cells, and the level of dTTP was substantially lower (30-35 times). Thus, in the M/M populations, the ratio of the ddNTP to the corresponding normal dNTP was equal or greater than that found in T cells. There are two known mechanisms by which ddNTP inhibit the replication of HIV (and other retroviruses) (18, 21, 45, 46). One is that they act as competitive inhibitors of the corresponding dNTP at the level of HIV's DNA polymerase (reverse transcriptase) with K_i of 0.04-0.2 μM (18, 21-23). The major cellular DNA polymerase (DNA polymerase α) is rather resistant to these drugs (47). In addition, because of the replacement of their 3'-hydroxy group with hydrogen or another group, once these compounds are incorporated at the end of a growing chain of viral DNA, no further nucleotide residues can be added. Thus they can act as chain terminators after having elongated a growing chain of DNA by one residue (18, 21, 45). It therefore follows that the activity of ddN in a given cell will be heavily affected by the ratio of the ddNTP level to the normal endogenous dNTP level, and this may account for the activity of AZT in M/M in spite of the limited phosphorylation of this drug.

M/M are a heterogeneous population, and one must be cautious in extrapolating the results here to other populations of these cells. Certain subsets of M/M and/or M/M belonging to the same subpopulation but at different stage of maturation could exhibit variability in HIV replication, drug activation, or both. We have tried in part to address this by examining M/M prepared by several methods (including cells allowed to adhere and differentiate for 5 d). Nevertheless, it is still possible that HIV replication in certain differentiated macrophages may be inhibited only by higher concentrations of ddN, and that not all cells within our heterogeneous populations are rendered virally resistant by these agents.

In this regard, it is worth pointing out that Richman et al. (24) have recently reported that AZT, ddC, and ddA failed to inhibit HIV infection of monocytes prepared by 3-7-d adherence (24). There are a number of differences in the experimental techniques used by that group and those used by us in this study; for example, Richman et al. used HIV_{LAV-1} (a T cell-tropic strain), did not preincubate the cells with drugs, and examined protection after 3 d in culture (24), whereas we used a

monocytotropic strain of the virus, preincubated the cells with drugs for 20 min before viral infection, and examined the antiviral effect over a 28-d period. It is possible as well that subtle differences may have affected the M/M populations in these two studies and influenced the results. It has been shown that M/M digesting cellular debris, for example, normally excrete nucleosides (such as thymidine) (44). Thymidine can outcompete (nullify) the antiviral activity of AZT (13, 32). At the same time, after anabolic phosphorylation, thymidine can potentiate the activity of ddC, in part by complex allosteric effects acting at the level of ribonucleotide reductase, to reduce cellular dCTP pools (48). It is thus conceivable that in crowded cultures of macrophages actively phagocytosing cellular debris, there may be a variable pattern of antiviral effect induced by ddN, leading to different observations and conclusions. Finally, our current data do not address subtle perturbations of accessory cell function that theoretically could occur even though an ongoing state of viral replication did not exist.

Our results do suggest, however, that HIV replication in fresh peripheral blood M/M can be efficiently suppressed by AZT, ddC, or ddA, and that certain differentiated (5-d-cultured) populations can also be rendered resistant to viral replication. It is unlikely that these results were due to contaminating T cells in the populations because (a) our populations contained <1% T cells, (b) we did not stimulate the cells with IL-2 or PHA, and (c) we used a monocytotropic strain of HIV that infects T cells less efficiently than M/M. The results suggest, at minimum, that the peripheral blood monocytes, which may disseminate HIV through the immune system, can be protected against HIV infection by ddN under some conditions. These results may also have implications in understanding the effects of these drugs on virus in the central nervous system. We have found that HIV-induced dementia may be reversed in certain patients by the administration of AZT (49, 50). It is possible that the protection of M/M in the central nervous system (e.g. microglial cells) by AZT may have contributed to the clinical improvement in these patients.

As a final point, however, it should be reemphasized that ddN do not substantially reduce HIV expression in T cells that are already infected (Mitsuya et al., unpublished data). We do not know how long infected M/M produce virus in vivo, but such long-lived infected cells may pose a problem for antiretroviral therapy even if de novo infection and recruitment of new cells can be blocked. Further study of the patterns of infection of M/M in vivo will thus be important in designing therapeutic strategies for the antiviral therapy of HIV infection.

Summary

Because of the probable role of HIV-infected monocyte/macrophages in the pathogenesis and progression of AIDS, it is essential that antiretroviral therapy address viral replication in cells of this lineage. Several dideoxynucleosides have been shown to have potent in vitro and, in the case of 3'-azido-2',3'-dideoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC), in vivo activity against HIV. However, because these compounds must be phosphorylated (activated) in target cells, and because monocyte/macrophages may have levels of kinases that differ from those in lymphocytes, we investigated the capacity of these drugs to suppress HIV replication in monocyte/macrophages using HIV-1/HTLV-III_{Ba-L} (a monocytotropic isolate). In the present study, we observed that HTLV-III_{Ba-L} replication in fresh human periph-

eral blood monocyte/macrophages was suppressed by each of three dideoxynucleosides: 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxyadenosine (ddA). Similar results were observed in 5-d-cultured monocyte/macrophages, although higher concentrations of the drugs were required. We then studied the metabolism of AZT and ddC in such cells. The phosphorylation of ddC to a triphosphate moiety was somewhat decreased in monocyte/macrophages as compared with H9 T cells. On the other hand, the phosphorylation of AZT in monocyte/macrophages was markedly decreased to 25% or less of the level in T cells. However, when we examined the level of the normal endogenous 2'-deoxynucleoside triphosphate pools, which compete with 2',3'-dideoxynucleoside triphosphate for viral reverse transcriptase, we found that the level of 2'-deoxycytidine-triphosphate (dCTP) was six- to eightfold reduced, and that of 2'-deoxythymidine-triphosphate (dTTP) was only a small fraction of that found in T cell lines. These results suggest that the ratio of dideoxynucleoside triphosphate to normal deoxynucleoside triphosphate is a crucial factor in determining the antiviral activity of dideoxynucleosides in HIV target cells, and that the lower levels of dTTP may account for the antiretroviral activity of AZT in the face of inefficient phosphorylation of this compound.

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