***Brief Definitive Report***

# FAILURE OF DIDEOXYNUCLEOSIDES TO INHIBIT HUMAN IMMUNODEFICIENCY VIRUS REPLICATION IN CULTURED HUMAN MACROPHAGES

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Human immunodeficiency virus (HIV) is the etiologic agent of the acquired immunodeficiency syndrome (AIDS). The cytolytic infection of CD4 lymphocytes by HIV (1, 2) contributes to progressive immunodeficiency. More recently, CD4 monocyte/macrophages have been shown to be infected in vitro (3, 4) and in vivo (5–10). These cells may serve as a reservoir for the virus because HIV replication in monocyte/macrophages appears to be more prolonged and less cytolytic than in lymphocytes (3, 4, 8).

The most potent inhibitors of HIV replication yet identified are dideoxynucleosides (11, 12), which include 3′-azido-3′-deoxythymidine (AZT, azidothymidine, zidovudine), 2′,3′-dideoxycytidine (ddC), and 2′,3′-dideoxyadenosine (ddA). To become active, these drugs are phosphorylated by host cells, and as 5′-triphosphates inhibit the viral reverse transcriptase and/or terminate DNA chain elongation (13, 14). AZT has been evaluated in patients with AIDS and AIDS-related complex (ARC), and has been shown to increase CD4 cell counts, improve cutaneous delayed hypersensitivity, and reduce the frequency of serious opportunistic infections; however, it does not significantly reduce the rate of recovery of virus from peripheral blood of treated patients. (15–17). ddC is under phase I investigation.

Previous studies of dideoxynucleosides in vitro have used either a mixed population of human peripheral blood mononuclear cells or continuously dividing CD4 cell lines. We now report the activity of these drugs in terminally differentiated, nondividing, primary human monocyte-derived macrophages (MDM). Compared to T lymphoblastoid cells, the MDM were found to have diminished deoxynucleoside kinase activities and reduced ability to phosphorylate AZT, ddC, or ddA. None of these dideoxynucleosides inhibited the infection or replication of HIV in macrophages in vitro.

## Materials and Methods

*Cells.* The human T lymphoblastoid cell line, CCRF-CEM, and the human T cell leukemia virus type 1–transformed human T lymphoblastoid cell line, MT2 (18), were

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obtained and propagated in RPMI 1640 medium containing 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FCS (Hyclone Laboratories, Logan, UT).

Human MDM cultures were obtained from the blood of volunteers who were negative for antibody to HIV. Mononuclear cells were prepared using isopycnic centrifugation in ficoll-Hypaque (4).

For infectivity studies, the mononuclear cells were suspended at $10^6$ cells/ml in RPMI 1640 with 2 mM L-glutamine, 50 μg/ml gentamicin, and 10% autologous serum, and 1.0 ml was placed in the 1-$cm^2$ wells of a 48-well plate (Costar, Cambridge, MA). After incubation at 37°C in 5% $CO_2$ for 3–7 d, nonadherent cells were removed by gently washing the monolayers three times with warm media. At this point the medium was modified to contain 10% FCS instead of autologous serum. Cells prepared by this technique were at least 95% MDM, as judged by esterase staining. For nucleoside kinase and uptake studies, the MDM were separated from peripheral blood lymphocytes by two cycles of adherence to gelatin-coated flasks (19).

*Drugs.* AZT and [$^3$H]AZT were the generous gifts of S. Lehrman, P. Furman, and T. Krenitsky (Burroughs-Wellcome, Research Triangle Park, NC). ddC and [$^3$H]ddC were the generous gifts of I. Sim (Hoffman-LaRoche, Nutley, NJ). ddA was purchased from Sigma Chemical Co. (St. Louis, MO). [$^3$H]ddA was purchased from Moravek Biochemicals, Brea, CA. The radioactive and unlabelled dideoxynucleosides were mixed to yield a final specific activity of 1–2 mCi/μmol.

*Nucleoside Kinase and Uptake Assays.* For nucleoside kinase assays, cells were washed at 4°C in isotonic PBS, pH 7.4, and then were frozen and thawed three times. After centrifugation and desalting on Sephadex G-25, nucleoside kinase activities were measured radiochemically (20) at a final substrate concentration of 10 μM. The reaction mixtures contained 10 mM ATP, 10 mM $MgCl_2$, 10 mM NaF, and cellular protein (10–40 μg), in 50 mM Tris, pH 8.0. After 10- and 30-min incubations, the nucleotide products were separated by thin-layer chromatography on polyethyleneimine-cellulose in methanol/water (1:1). Activities are expressed as picomoles per minute per milligram protein, determined according to Bradford (21).

For nucleoside uptake studies, cells were suspended at a density of $2 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine and either 10% autologous plasma or serum (for MDM), or 10% FCS (for CEM). The labelled nucleosides (1 μM, 1.0 μCi/ml) were added to the cell suspensions, which were incubated in 17 × 100-mm polypropylene tubes at 37°C, with occasional shaking. After 4 or 18 h, the cells were washed three times with PBS until the supernatants were free of radioactivity. Then an aliquot of ddATP or ddCTP was added as an internal recovery marker. The cell pellets were extracted with 60% methanol at −20°C overnight. The radioactivity in the solubilized pellets was determined in a liquid scintillation counter. The nucleotides in some of the samples were fractionated by HPLC (13, 14).

*Virus.* The LAV-1 strain of HIV was obtained from Drs. F. Barre-Sinoussi, J.-C. Chermann, and L. Montagnier (Institut Pasteur, Paris, France). A cell-free virus pool was prepared in CEM cells and had a titer of $10^8$ $TCID_{50}$/ml as assayed by giant cell formation in MT2 cells.

*Antiviral Drug Assays.* Cells were infected at a multiplicity of infection of one tissue culture 50% infectious dose $TCID_{50}$/cell for 60 min at 37°C in medium containing 1% polybrene. MDM cell monolayers containing ~$10^5$ cells/well in 24-well plates were then washed three times before incubation in medium containing drug. CEM cells were infected in suspension at $6 \times 10^6$ cells/ml, washed three times by centrifugation and resuspension, and then distributed in 48-well plates at $6 \times 10^5$ cells/well before the addition of medium containing drug. Antiviral activity was assayed by the inhibition of the production of HIV p24 (*gag*) antigen in the cell-free culture medium of infected cells exposed to different concentrations of drugs. p24 antigen was assayed by ELISA (Abbott Laboratories, Chicago, IL) according to the manufacturer's instructions. The postwash antigen levels were <100 pg/ml.

## Results

*Nucleoside Kinase Activities.* The nucleoside kinase activities in extracts of human MDM and CEM T lymphocytes were assessed radiochemically (Table I).

TABLE I
*Nucleoside Kinase Activities*

| Cells | Thymidine kinase | Deoxycytidine kinase | Uridine kinase | Adenosine kinase |
|---|---|---|---|---|
| CEM T lymphoblasts | 36 ± 16 | 20 ± 8 | 113 ± 59 | 332 ± 74 |
| MDM | 9 ± 4 | 4 ± 2 | 13 ± 8 | 202 ± 78 |

The nucleoside kinase activities in extracts of CEM T lymphoblasts or MDM were determined radiochemically at a substrate concentration of 10 μM. Activities are expressed as picomoles of nucleotide per minute per milligram protein (±SEM; $n = 3$ for CEM, $n = 4$ for MDM).

TABLE II
*Nucleoside Phosphorylation by Intact Cells*

| Cells | AZT | ddC | ddA |
|---|---|---|---|
| CEM T lymphoblasts | 17 ± 2 | 2.8 ± 1.6 | 2.8 ± 0.5 |
| MDM | 2 ± 0.8 | 1.5 ± 0.6 | 1.6 ± 0.1 |

Either CEM T lymphoblasts or MDM were incubated for 4 h with 1 μM radiolabelled AZT, ddC, or ddA. After washing and extraction, radioactive nucleotide formation was assessed. The results are expressed as picomoles per $10^6$ cells (±SEM; $n = 3$ for CEM, $n = 2$ for MDM), and are corrected for recoveries of 60–90%.

Compared to the T lymphoblasts, the MDM had lower levels of thymidine kinase, deoxycytidine kinase, and uridine kinase. Adenosine kinase activity was abundant in both cell types.

*Phosphorylation of Dideoxynucleosides.* MDM and CEM cells were compared for their ability to phosphorylate dideoxynucleosides (Table II). CEM T lymphoblasts accumulated more of each dideoxynucleotide over a 4-h period than did MDM. The disparity persisted when the incubations were prolonged to 18 h. The difference in phosphorylation between T lymphoblasts and MDM was much greater for AZT than for either ddC or ddA. HPLC analyses revealed that, in both cell types, 5′-triphosphates accounted for ~10% of AZT nucleotides and 2% of ddC nucleotides.

*Inhibition of HIV Replication by Dideoxynucleosides.* The relative antiviral effects of the dideoxynucleosides in the CEM CD4 lymphoblastoid cell line and in MDM cells were compared (Fig. 1). In CEM cells, >50% inhibition of p24 antigen is observed with 0.1 μM ddC, 1 μM AZT, or 10 μM ddA. These results correspond to those obtained by others in different lymphocyte-derived cell lines or in stimulated primary human lymphocyte cultures (11, 12). In contrast, little if any antiviral activity could be documented in MDM cells with any of these three drugs, at concentrations as high as 100 μM. Similar results were obtained in nine separate experiments, and for cells treated with drugs before infection.

## Discussion

The CD4 lymphocyte is well-recognized as a critical host cell in the pathogenesis of HIV infection. Dideoxynucleosides are phosphorylated by these cells, and the triphosphate products have potent antiviral activity (11–14). HIV has also been found to replicate in macrophages. Cells with macrophage markers in the

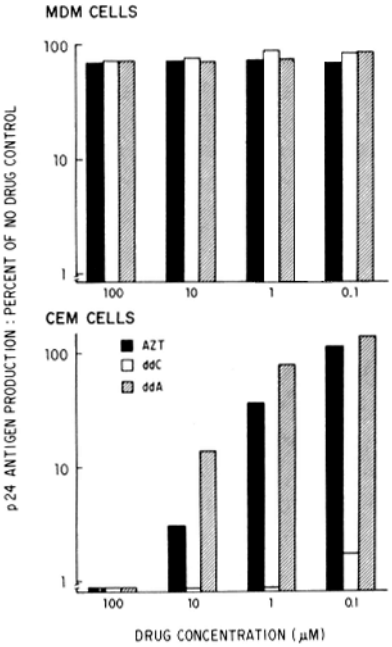


FIGURE 1. Inhibition of p24 antigen production in the medium of HIV-infected cell cultures exposed to various concentrations of dideoxynucleosides. HIV antigen levels were measured in the cell-free medium of cultures 72 h after infection at a multiplicity of infection of 1 $TCID_{50}$/cell. Each value was assayed on triplicate wells. The values for the no-drug control wells were 1,585 ± 135 ng/ml for CEM cells and 1,200 ± 158 pg/ml for MDM cells.

brains, lungs, and skin of patients with AIDS have been shown to contain infectious virus, viral antigens, or viral nucleic acid (3–9). Human MDM cells can be readily infected in vitro (3, 4).

In this report, human MDM were shown to have lower deoxynucleoside kinase activities than T cells, and to phosphorylate three dideoxynucleosides with anti-HIV activity (AZT, ddC, and ddA) less efficiently than T lymphocytes. The MDM permitted HIV replication at dideoxynucleoside concentrations that were 10–1,000-fold higher than those sufficient to block HIV infection of T lymphoblasts. Although MDM accumulated less dideoxynucleoside 5′-triphosphates than did T lymphoblasts over a 4-h period, the magnitude of the difference cannot account entirely for the failure of the dideoxynucleosides to inhibit HIV replication in MDM. Other factors, such as nucleotide pools and the initial rate of nucleotide formation compared with the time of reverse transcription, may also influence the anti-HIV activity of the compounds in MDM.

Murine (22, 23) and human (24) macrophages have been reported to lack thymidine kinase activity. These cells, in fact, release deoxypyrimidines extracellularly, an activity that has been proposed to modulate lymphocyte function (22–24). It is not surprising that a cell no longer programmed to synthesize DNA has reduced requirements for deoxynucleoside salvage. These cells, which are metabolically highly active and may survive for prolonged periods, do have high adenosine kinase activities, consistent with requirements for ATP synthesis.

The results of these studies may explain what have been confusing observations made as part of clinical trials of AZT. The treatment of patients with AIDS or ARC with AZT has resulted in elevation of CD4 peripheral blood cell counts, restoration of cutaneous delayed hypersensitivity, and reduction in the rate of opportunistic infections and death (15, 16). However, AZT had no effect on virus isolation rates from peripheral blood cells, despite a reduction of circulating

p24 antigen in serum (16). These observations suggest that a subset of infected cells persists which represent a reservoir of continuing viral replication. Perhaps the infection of macrophages in vivo by HIV cannot be prevented completely by dideoxynucleosides. This conclusion suggests that more effective therapy of HIV infection may require either the delivery of prephosphorylated nucleosides, perhaps via liposomes, or the identification of compounds whose antiviral activity is independent of host cell metabolism.

## Summary

Primary human monocyte-derived macrophages (MDM) were shown to have diminished deoxynucleoside kinase activities compared to T lymphoblasts, and a reduced ability to phosphorylate dideoxynucleosides with anti–human immunodeficiency virus (HIV) activity. These drugs, azidothymidine (AZT), dideoxycytidine (ddC), and dideoxyadenosine (ddA), which are potent anti-HIV agents in CD4 lymphocytes, did not inhibit HIV replication in MDM, even at concentrations of 100 $\mu$M. This drug concentration of AZT is ~100-fold higher than the levels attained in the serum of treated patients and the levels required to inhibit HIV replication in lymphocytes. These observations may explain the failure of AZT therapy to clear viremia, consistent with the presence of a drug-resistant reservoir of infected cells in vivo. New therapeutic approaches to inhibit the replication of HIV in MDM may be needed.

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