REPLICATION OF HUMAN IMMUNODEFICIENCY VIRUS IN MONOCYTES

Granulocyte/Macrophage Colony-stimulating Factor (GM-CSF) Potentiates Viral Production yet Enhances the Antiviral Effect Mediated by 3'-Azido-2'3'-dideoxythymidine (AZT) and Other Dideoxynucleoside Congeners of Thymidine

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Human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS), infects and replicates within several types of human cells (1–4). Although early studies of this retroviral pathogen focused on $CD4^+$ T lymphocytes as the principal target cell, there is increasing evidence that infection of cells belonging to the monocyte/macrophage (M/M)[1] lineage plays a crucial role in the pathogenesis and progression of this disease (2, 5, 6). In addition, since M/M may differ from T cells in their intracellular metabolism of drugs and their reaction to lymphokines, it is important that one consider the effect of antiretroviral therapeutic strategies on cells of the M/M lineage.

Our group has previously shown that 2'3'-dideoxynucleosides (ddN), a family of compounds lacking an hydroxy group at the 3' position of the sugar moiety, are potent in vitro inhibitors of HIV replication in both human T lymphocytes and M/M (7–9). In addition, several members of this family of drugs have been shown to have clinical activity when administered to patients with severe HIV infection (10–12). One of these compounds, 3'-azido-2'3'-dideoxythymidine (AZT) prolongs life and improves neurologic abnormalities (10, 12–14) in certain patients with AIDS. The use of AZT, however, is associated with bone marrow suppression and other toxic side effects (15), particularly in patients with established AIDS, so that a recent focus of investigation has been directed toward developing strategies to overcome this problem. One

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[1] *Abbreviations used in this paper:* AZddU, 3'-azido-2'3'-dideoxyuridine; AZT, 3'-azido-2'3'-dideoxythymidine; AZTTP, 3'-azido-2'3'-dideoxythymidine-5'-triphosphate; dCTP, 2'-deoxycytidine-5'-triphosphate; ddA, 2'3'-dideoxyadenosine; ddC, 2'3'-dideoxycytidine; ddI, 2'3'-dideoxyinosine; ddT, 2'3'-dideoxythymidine; D4T, 2'3'-dideoxy-2'3'-didehydrothymidine; dTTP, 2'-deoxythymidine-triphosphate; dUMP, 2'-deoxyuridine-5'-monophosphate, GM-CSF, Granulocyte-macrophage colony stimulating factor; M/M, Monocyte/macrophages; RT, reverse transcriptase.

approach currently under investigation is the use of granulocyte-macrophage CSF (GM-CSF). GM-CSF is a cytokine that stimulates the maturation and differentiation of granulocyte and monocyte bone marrow precursor cells (16–18). In addition, Hammer et al. (19, 20) have suggested that GM-CSF can inhibit HIV replication in U937, a $CD4^+$ cell line with certain monocytoid properties. However, Folks et al. (21) have found that GM-CSF may actually stimulate HIV replication in a subclone of U937.

Because of the interest in using GM-CSF in patients with HIV-related disease and the conflicting results obtained in monocytoid cell lines, which may or may not be reliable biochemical models for normal M/M, we undertook an investigation of the in vitro effect of GM-CSF on HIV replication in purified human peripheral blood M/M, both as a single agent and in conjunction with dideoxynucleosides. Our results show that GM-CSF enhances HIV replication in fresh M/M by several hundred-fold, and moreover, that infection of such stimulated cells can take place with lower concentrations of virus. In spite of this, the net potency of AZT and related dideoxythymidine analogues in inhibiting HIV infection is markedly increased in the presence of GM-CSF. This increase in activity appears to result from an enhanced cell entry of drug and production of phosphorylated (activated) drugs in the face of minimal increases in the levels of the competing thymidine triphosphate. These results may have implications for the design of improved therapeutic strategies for patients with AIDS and related conditions.

Materials and Methods

Cells. Monocyte-enriched PMBC were obtained from healthy, HIV-negative donors using a cell separator (Fenwal C3000; Travenol Inc., Deerfield, IL). The PBMC were then further enriched for M/M by elutriation as described by Gerrard et al. (22). M/M obtained by elutriation followed by a 5-d culture were >95% nonspecific esterase positive (Technicon Instruments Corp., Tarrytown, NY), and >95% actively phagocytized 0.8-μ latex beads (Sigma Chemical Co., St. Louis, MO). In addition, they were <1% E-rosette positive, and 50% $OKT4A^+$ (Ortho Diagnostic Systems Inc., Raritan, NJ). Cell viability (as determined by trypan blue exclusion) was always >95%. In some experiments, H9, a $T4^+$ lymphocytic cell line, was used as control. Also, in certain experiments, enriched lymphocyte populations were obtained by elutriation followed by incubation on plastic dishes to remove adherent cells. These cells were <2% nonspecific esterase positive. It should be noted that the control M/M used in this study (i.e., elutriated cells cultured for 5 d before exposure to HIV) are different than the M/M populations that formed the core of our previous paper on the effect of dideoxynucleosides on HIV infection in M/M (8).

Virus. A monocytotropic strain of HIV-1, $HTLV\text{-}III_{Ba\text{-}L}$ (kindly provided by Drs. S. Gartner and M. Popovic, National Cancer Institute, Bethesda, MD), and a lymphocytotropic strain, $HTLV\text{-}III_B$ (Electro-Nucleonics Laboratory Inc., Silver Spring, MD), were used in these experiments, as previously described (8).

Drugs. AZT (Wellcome Research Laboratories, Research Triangle Park, NC), 2′3′-dideoxycytidine (ddC), 2′3′-dideoxythymidine (ddT), 2′3′-dideoxyadenosine (ddA), 2′3′-dideoxyinosine (ddI) (Pharmacia Fine Chemicals, Piscataway, NJ), 2′3′-dideoxy-2′3′-didehydrothymidine (D4T) (supplied by Developmental Therapeutics Program, DCT, NCI, Bethesda, MD), and 3′-azido-2′3′-dideoxyuridine (AZddU) (kind gift of Dr. R. F. Schinazi, VA Medical Center, Decatur, GA) were diluted in distilled water and kept at 4°C until used. The chemical structures of AZT, AZddU, ddT, and D4T are depicted in Fig. 1. Human recombinant GM-CSF (Sandoz Research Inst., East Hanover, NJ) was reconstituted in distilled water at 500,000 U/ml stock solution, and stored at 4°C until used. Our GM-CSF preparation contains 5.6×10^6 chronic myelogenous leukemia (CML) units per milligram of glycoprotein, as mea-

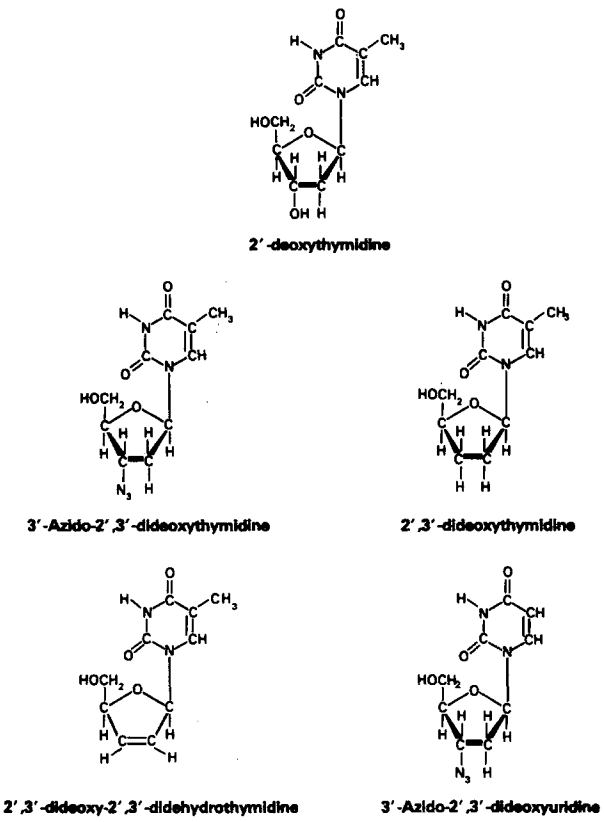

FIGURE 1. Chemical structure of 2'-deoxythymidine (dT) and its dideoxynucleoside congeners, AZT, ddT, D4T, and AZddU. The term 2'-deoxythymidine is synonymous with the term thymidine.

sured by minor modifications of a rapid proliferation CML assay previously published by Griffin et al. (23).

[^{3}H]AZT (sp act 3 Ci/mmol), and [^{3}H]D4T (sp act 10 Ci/mmol) (Moravek Biochemical, Brea, CA), were freeze-dried and used immediately after reconstitution.

Antiviral Drug Assay. The tests of antiviral activity were performed with minor modifications of a previously published procedure (8). Briefly, 10^5 purified elutriated M/M were seeded at day −5 in a 1-cm^2 well of a 48-well plate (Costar, Cambridge, MA), and cultivated in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 20% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco Laboratories) (complete medium) for 5 d in the presence or absence of 100 U/ml of GM-CSF. Unless stated otherwise, cells were treated from day −5 with GM-CSF and then cultured in the continuous presence of this cytokine. Cells, viruses, and FCS were tested for mycoplasma contamination, and found negative. At day 0, M/M were preexposed for 20 min to various concentrations of drugs, and then challenged with HTLV-III$_{Ba-L}$ (80,000 cpm/ml reverse transcriptase) as previously described (8). This is at least 10 times the minimum infective dose in 5-d cultured M/M not exposed to GM-CSF (see Results, Fig. 2 *b*). Cells were kept at 37°C in a humidified atmosphere supplemented with 5% CO_2. 2 d after infection (day +2) cells were extensively washed in order to remove excess virus, and cultivated under the same conditions with the same concentrations of drugs as before. Cells were then fed every 4–5 d. In some experiments, purified lymphocytes stimulated at day 0 with PHA, 1:200 vol/vol (Gibco Laboratories), or H9 T cells were exposed to 500 viral particles/cell of HTLV-III$_B$ (500 times the minimum infective dose

for ATH8 cells, a T4 cell clone used as virus infectivity control). 20 U/ml of human rIL-2 (AmGen Biological, Thousand Oaks, CA) plus 15% vol/vol of human natural IL-2 (ABI, Silver Spring, MD) were added to medium for lymphocytes (but not for other cells).

Viral Detection. Viral production was assessed at days 2, 5, and 7 after viral challenge, and then every 7 d, using several different methods. (*a*) reverse transcriptase (RT) activity was evaluated using a previously described procedure (8, 24). (*b*) HIV-p24 antigen production into the supernatant was assessed by a RIA and by ELISA (DuPont Co., Wilmington, DE). (*c*) Syncytia formation was evaluated at regular time points by visual inspection in an inverted microscope.

Drug toxicity was assessed in mock-infected cells cultivated for 14 d in presence of different concentrations of ddN, by trypan blue exclusion, and by phagocytic activity using 0.8-μ latex beads.

Metabolism. Anabolic phosphorylation of dideoxynucleosides was analyzed using minor modifications of previously published procedures (25, 26). Briefly, ddN metabolism was measured by incubating 5×10^6 cultivated M/M or H9 T cells, in the presence or absence of 100 U/ml of GM-CSF, in 12-well plates (Costar). After 5 d of culture, cells were exposed to 10 μM [^{3}H]AZT or 10 μM [^{3}H]D4T for 24 h in 4 ml of complete medium. Cells were then harvested by gentle scraping, counted, pelleted after three washings in cold RPMI, and immediately frozen in dry ice. Frozen cell pellets were extracted with 500 μl of 60% (vol/vol) methanol, and the methanol extracts further heated at 95°C for 1.5 min. The extracts were clarified by centrifugation at 12,000 *g* for 6 min. 200-μl aliquots were then loaded onto a radially compressed column of Partisil SAX and eluted with an ammonium phosphate gradient as previously described (24, 25). Endogenous deoxyribonucleoside-5′-triphosphate pools and nucleoside kinases were measured as previously described (27).

Statistics. The statistical significance of the difference of viral suppression by dideoxynucleosides in GM-CSF-stimulated cultures vs. cultures without GM-CSF was assessed using the two-tailed student's *t* test.

Results

Fresh elutriated M/M cultured for 5 d before exposure to HTLV-III$_{Ba-L}$ were consistently found to be infected in these experiments (Fig. 2). In the absence of GM-CSF, viral production was detectable no earlier than day 7 and then increased, being detectable at least up to day 35 (Fig. 2 *A*). It should be noted that the M/M population and the culture conditions used are critical variables in experiments such as these, since the degree of HIV infection and viral suppression by dideoxynucleosides may vary substantially depending on the exact M/M population and conditions (8). Culturing the elutriated M/M for 5 d before exposure to virus and drugs is thus an important variable that should be taken into consideration.

M/M exposed continuously to 100 U/ml GM-CSF showed a high titer of viral production (at least 10 times more than without this cytokine), consistently detectable by day 5. Viral production was sustained for at least 35 d. No substantial enhancement of viral production was seen in most experiments using lower concentrations of GM-CSF, i.e., 1 and 10 U/ml (data not shown). Since GM-CSF induces proliferation of monocytes, we wondered whether the increase in viral production was simply due to the GM-CSF-induced increase in cell number. However, the number of cells 7 d after viral challenge ranged up to 20-fold higher for M/M exposed to GM-CSF as compared with unexposed cells, whereas the increase in p24 production at day 7 was ~100–1,000-fold. (Table I and Fig. 2 *A*). Furthermore, in early experiments, we tested the minimum viral dose capable of inducing consistent and productive infection in M/M. We found that while the minimum HTLV-III$_{Ba-L}$ infective dose for untreated M/M was 8,000 cpm/ml of RT, as little as 80 cpm/ml of

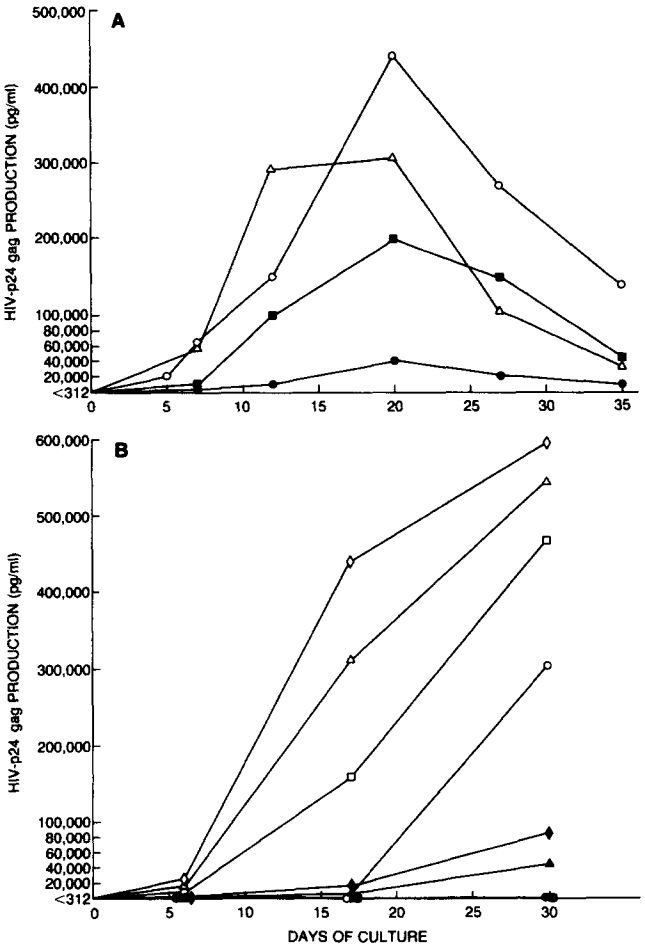

FIGURE 2. Kinetics of HTLV-III$_{Ba-L}$ infection in M/M with or without GM/CSF 100 U/ml. (*A*) HIV-p24 gag production (pg/ml) in M/M exposed at day 0 to 80,000 cpm RT/ml of HTLV-III$_{Ba-L}$ and cultured in complete medium as described. Supernatants were harvested for p24 assays every 4–7 d. At each of these time points, cells were washed, and fresh media was added. (●) M/M without GM-CSF; (○) M/M exposed to GM-CSF from day −5 to day 28; (△) M/M exposed to GM-CSF from day −5 to day 0; (■) M/M exposed to GM-CSF from day 0 to day 28. (*B*) HIV-p24 gag production (pg/ml) in M/M exposed to different multiplicity of infection of HTLV-III$_{Ba-L}$. Open symbols represent M/M treated with GM-CSF from day −5 to day 30. Closed symbols represent untreated M/M. (◇/◆) M/M exposed to 80,000 cpm RT/ml; (△/▲) M/M exposed to 8,000 cpm RT/ml; (□/■) M/M exposed to 800 cpm RT/ml; (○/●) M/M exposed to 80 cpm RT/ml.

the same virus preparation induced viral infection in GM-CSF-treated M/M (Fig. 2 *B*). Thus, it is unlikely that the increase in cell number per se caused by GM-CSF accounts for the major enhancement of viral production observed in our studies. Syncytia formations were easily detected, starting from day 9, in GM-CSF-M/M exposed to HTLV-III$_{Ba-L}$ (Fig. 3). At this time, multinucleated giant cells were seen in culture, in association with an inhibition of cell replication. From day 20, giant cells became pycnotic. However, they maintained their ability to produce a large amount of virus at least up to day 35. In one experiment, we subjected HIV-exposed GM-CSF-stimulated M/M to three cycles of freezing and thawing to explore whether this would release additional viral particles that might be sequestered within intracytoplasmic vacuoles; however no increase in HIV-p24 antigen was observed (data not shown).

As shown in Fig. 2 *a* and in Table I, M/M treated with GM-CSF only before virus challenge (that is from day −5 to day 0), followed by extensive washing to remove this cytokine, initially gave rates of viral production comparable with those

TABLE I

Virus Production and Viral Suppression by AZT in Elutriated M/M Treated with GM-CSF or Media Alone at Different Time Points and Exposed to HIV-1/HTLV-III$_{Ba-L}$

Day after infection	AZT	No GM-CSF	GM-CSF (day −5 to day 28)	GM-CSF (day −5 to day 0)	GM-CSF (day 0 to day 28)
	μM				
7	0	70	61,703	51,100	8,000
	0.1	60 (14%)	<31 (100%)	<31 (100%)	<31 (100%)
	1	<31 (>56%)	<31 (100%)	<31 (100%)	<31 (100%)
	10	<31 (>56%)	<31 (100%)	<31 (100%)	<31 (100%)
14	0	9,800	144,500	305,000	59,000
	0.1	7,500 (23%)	<31 (100%)	34 (99.9%)	<31 (100%)
	1	<31 (100%)	<31 (100%)	<31 (100%)	<31 (100%)
	10	<31 (100%)	<31 (100%)	<31 (100%)	<31 (100%)
28	0	21,000	265,400	119,100	137,100
	0.1	24,500 (0%)	445 (99.8%)	3,131 (97.3%)	65 (99.9%)
	1	185 (99.2%)	<31 (100%)	<31 (100%)	<31 (100%)
	10	<31 (100%)	<31 (100%)	<31 (100%)	<31 (100%)

10^5 fresh elutriated M/M were placed in culture at day −5 in 1 ml of complete medium, and treated with GM-CSF 100 U/ml from day −5 to 28, from day −5 to day 0, or from day 0 to 28. On day 0, M/M were exposed to saline or AZT for 20 min and challenged with HTLV-III$_{Ba-l}$ 80,000 cpm RT/ml while maintaining AZT in the media. Viral replication was assessed by HIV-p24 antigen production in the supernatants. The level of infection is expressed in pg/ml (numbers in parentheses show the percent of viral suppression). Results reported here represent a typical experiment.

seen in cells continuously exposed to GM-CSF. After day 14, however, a substantial waning in viral production was seen, and from day 35 after viral challenge this level became similar to the viral yield obtained in GM-CSF-unexposed M/M. Viral production in M/M treated with GM-CSF starting from day 0 was less enhanced than that observed with continuous treatment from day −5. In some experiments, we infected blood-derived M/M that had been allowed to mature for 5 d on plastic dishes, were further purified by adherence, and then exposed to GM-CSF. GM-CSF did not have any substantial effect on HIV replication in these 5-d adherent M/M (data not shown), possibly because of the relatively low number of high-affinity GM-CSF receptors in mature macrophages (28, 29). This is a topic for further research.

In similar experiments, we exposed elutriated M/M cultivated for 5 d in the presence or absence of GM-CSF to HTLV-III$_B$, a lymphocytotropic viral strain (Fig. 4). In agreement with our previous experience, in the absence of GM-CSF we obtained inconsistent viral replication using this strain; productive infection was attained in only three of six experiments using M/M not exposed to GM-CSF, and in the productive cultures there was a delayed, lower peak of viral production, as compared with the results obtained using the monocytotropic strain HTLV-III$_{Ba-L}$. However, HTLV-III$_B$ replication in M/M exposed to GM-CSF was consistent and easily detectable starting from 7 d after virus exposure, with a peak of viral production after 14–21 d of culture ~20 times greater than the maximum level obtained without GM-CSF. We also tried to define the minimum infective dose in these cells. As shown in Fig. 4, we observed that in the absence of GM-CSF, at least 100 times more

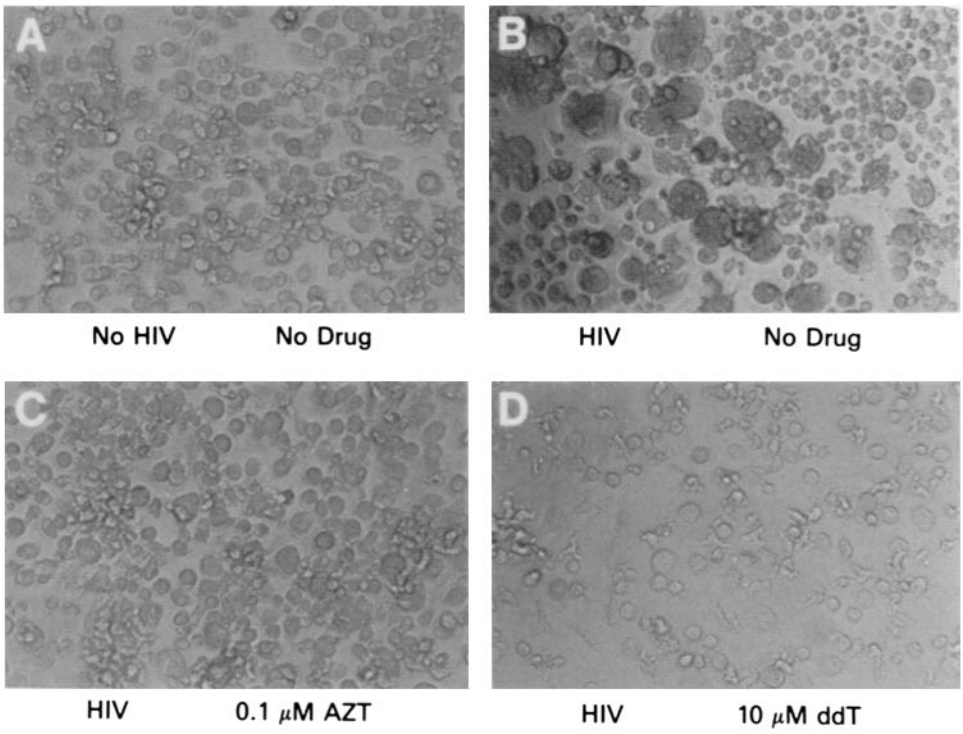

FIGURE 3. Syncytia formation at day 14 in GM-CSF-treated M/M exposed to HIV-1/HTLV-III_{Ba-L}. M/M were treated with GM-CSF 100 U/ml from day −5, then exposed at day 0 to HTLV-III_{Ba-L} 80,000 cpm RT/ml, in the presence or absence of 0.1 μM AZT or 10 μM ddT. (*A*) GM-CSF-treated M/M without HTLV-III_{Ba-L}; (*B*) GM-CSF-treated M/M exposed to HTLV-III_{Ba-L}; (*C*) GM-CSF-treated M/M exposed to HTLV-III_{Ba-L} in the presence of 0.1 μM AZT; (*D*) Same as *C*, except that M/M were cultured in the presence of 10 μM ddT.

HTLV-III_B was required to bring about a productive infection of M/M, compared with the dose of HTLV-III_B required to do so in the presence of GM-CSF. Thus, in our hands, GM-CSF induced in M/M an enhancement of HTLV-III_B replication in conjunction with a substantial reduction (at least 100-fold) of the minimum infective dose of HTLV-III_B, an otherwise nonmonocytotropic strain of HIV-1.

Also consistent with our previous report (8), AZT induced inhibition of HTLV-III_{Ba-L} replication in M/M precultivated for 5 d in media alone (Table I). Over 90% suppression of viral replication was obtained with 1 μM AZT, and 23% suppression was obtained with 0.1 μM. Interestingly, despite the substantial increase in HIV production induced in such cells by GM-CSF alone, AZT was much more potent in inhibiting viral replication in the presence of this cytokine (Fig. 5 *A*); >98% inhibition of viral replication was achieved with 0.1 μM AZT, and even with 0.01 μM AZT, ~90% viral suppression was observed (this was not due to an artifact of increased cell death, a point we will address later in this article). This effect was not seen in fresh, monocyte-depleted lymphocytes; in these cells, 10 μM AZT completely

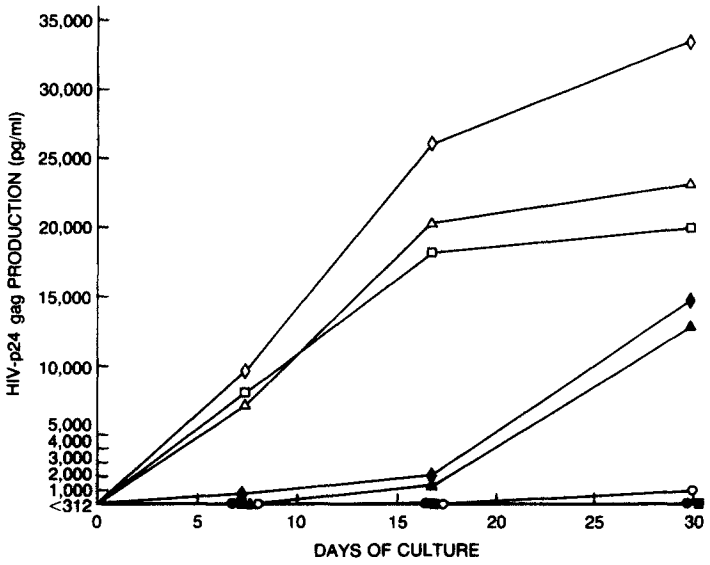

FIGURE 4. Kinetics of HTLV-III$_B$ infection in M/M exposed to GM-CSF 100 U/ml at day −5, and challenged at day 0 with different multiplicity of infection of the lymphocytotropic strain HTLV-III$_B$. Open symbols represent M/M treated with GM-CSF from day −5 to day 30. Closed symbols represent control M/M cultivated in media alone from day −5 to day 30. (◇/◆) M/M exposed to 10,000 HTLV-III$_B$ particles/cell; (△/▲) M/M exposed to 1,000 HTLV-III$_B$ particles/cell; (□/■) M/M exposed to 100 HTLV-III$_B$ particles/cell; (○/●) M/M exposed to 10 HTLV-III$_B$ particles/cell. Shown here is a representative experiment in which some detectable infection with HTLV-III$_B$ was attained in M/M not exposed to GM-CSF. While substantial viral replication was consistently achieved in GM-CSF-exposed M/M in all experiments, no detectable viral replication was obtained in three out of six experiments in M/M not exposed to GM-CSF.

inhibited HIV-1/HTLV-III$_B$ replication in the absence of GM-CSF, and neither stimulation of viral production nor enhancement of antiviral activity of AZT was seen with exposure of lymphocytes to 100 U/ml GM-CSF (data not shown). This is in agreement with the results obtained by Walker and Burgess (28), in which no substantial amount of GM-CSF receptors could be detected on T lymphocytes.

To determine whether GM-CSF enhancement of antiviral activity in M/M was specific for AZT, or was also seen with other 2′3′-dideoxy analogues of thymidine, we tested the effect of GM-CSF on three AZT congeners, ddT, D4T, and AZddU. Although AZddU is in fact a uridine congener, it is phosphorylated by thymidine kinase (30) and in this sense acts as congener of AZT. We also studied three other 2′3′-dideoxynucleosides, ddC, ddA, and ddI, drugs with different bases and which are phosphorylated in human cells by different kinases compared with AZT. The results obtained with the monocytotropic strain HTLV-III$_{Ba-L}$ are shown in Fig. 5. With each of the AZT congeners, there was an enhancement of antiviral activity in the presence of GM-CSF (Fig. 5 *A*). Consistent with our previous results, the ED$_{50}$ for AZT in elutriated M/M cultured for 5 d was ~0.1 μM, while it decreased down to between 0.001 and 0.01 μM when these M/M were exposed to GM-CSF. ddT itself induced complete viral inhibition at 10 μM in elutriated M/M, the ED$_{50}$ being 1 μM. This result is remarkable inasmuch as ddT does not have strong anti-

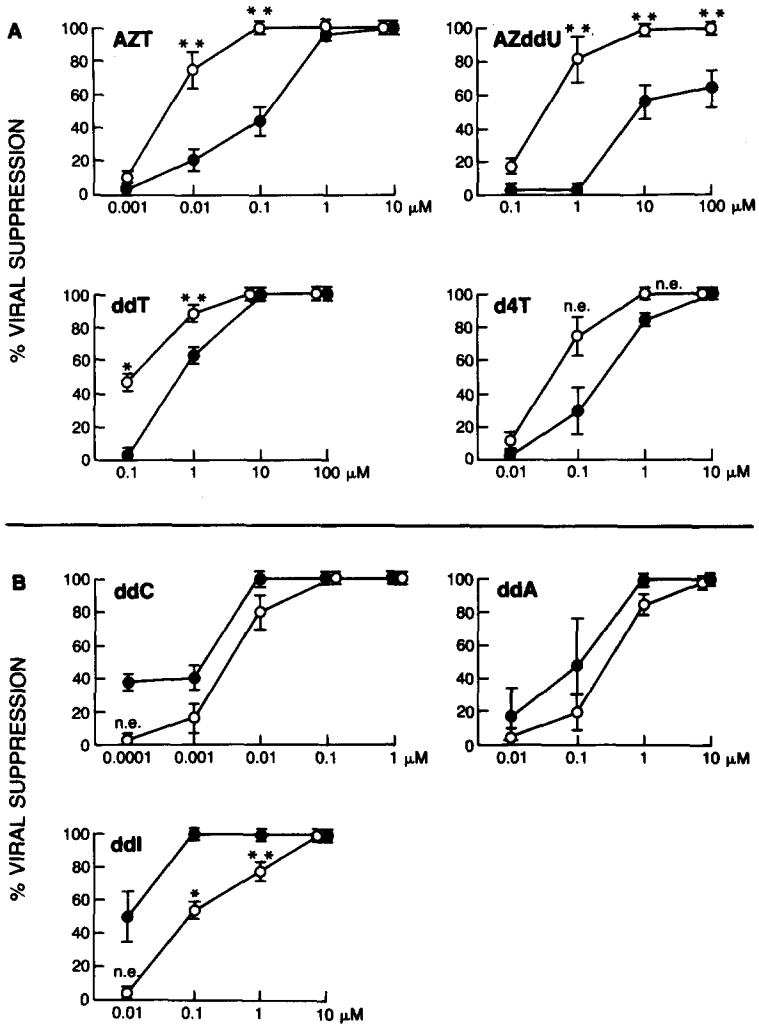

FIGURE 5. Viral suppression as a function of drug concentration in M/M treated with or without GM-CSF 100 U/ml from day −5 and exposed to HTLV-III$_{Ba-L}$ 80,000 cpm RT/ml in presence of various concentrations of ddN. (O) M/M treated with GM-CSF. (●) Control M/M not exposed to GM-CSF. (*A*) Percent of viral suppression in M/M exposed to four different dideoxynucleoside analogues of deoxythymidine as noted. (*B*) Percent of viral suppression in M/M exposed to dideoxynucleosides not related to deoxythymidine as noted. Viral replication was assessed by HIV-p24 gag production after 21 d of culture. Average control p24 gag production (without drugs) was as follows: M/M not treated with GM-CSF, 20,500 pg/ml; GM-CSF-treated M/M, 223,000 pg/ml. Results shown are the mean ± SEM of three or more separate experiments, except in the case of D4T and the lowest concentrations of ddC and ddI, which were evaluated in only two experiments. (*) $p < 0.05$; (* *) $p < 0.005$: (n.e.) p value not evaluated (only two experiments performed).

HIV activity in T cells in our hands (7). However, in the presence of GM-CSF, the ED_{50} was lowered to 0.1 μM. In a similar manner, the ED_{50} of D4T was between 0.1 and 1 μM for untreated M/M, and between 0.01 and 0.1 μM for GM-CSF-exposed M/M. Finally, AZddU was only partially effective in our hands in elutriated M/M

after 21 d of culture, even at the highest concentrations tested (~60% protection at 100 μM after 21 d of culture). However, 10 μM AZddU induced complete viral suppression in GM-CSF-treated M/M, with an ED_{50} of 0.1–1 μM. These data were confirmed in two different experiments by determination of RT activity in culture supernatants; the results were substantially parallel to those obtained measuring HIV-p24 antigen production (data not shown). We also tested the ability of AZT and its congeners to inhibit syncytia formation. As shown in Fig. 3, low concentrations of AZT and ddT completely inhibited syncytia induced by HIV infection in GM-CSF-exposed M/M; similar effects were seen with D4T and AZddU (data not shown).

We asked whether GM-CSF had an effect on viral infection and/or on antiviral activity of AZT in H9, a T lymphocytic cell line. Neither increase of viral production nor enhancement of antiviral activity of AZT and related congeners was seen, in agreement with data obtained with AZT in fresh lymphocytes (data not shown). Finally we exposed M/M to the lymphocytotropic strain $HTLV\text{-}III_B$ in the presence or absence of GM-CSF. The results, shown in Fig. 6, are substantially parallel to those obtained with the monocytotropic strain $HTLV\text{-}III_{Ba\text{-}L}$, showing an overall potentiation of viral suppression by AZT and related congeners in M/M exposed to GM-CSF. Thus, the results indicated that AZT and each of the closely related congeners tested are 10 or more times more active at suppressing replication of two different strains of HIV in GM-CSF-exposed M/M than in M/M not exposed to GM-CSF.

By contrast, when we tested the effect of GM-CSF on other dideoxynucleosides not related to AZT, we found that there appeared to be some reduction of anti-HTLV-

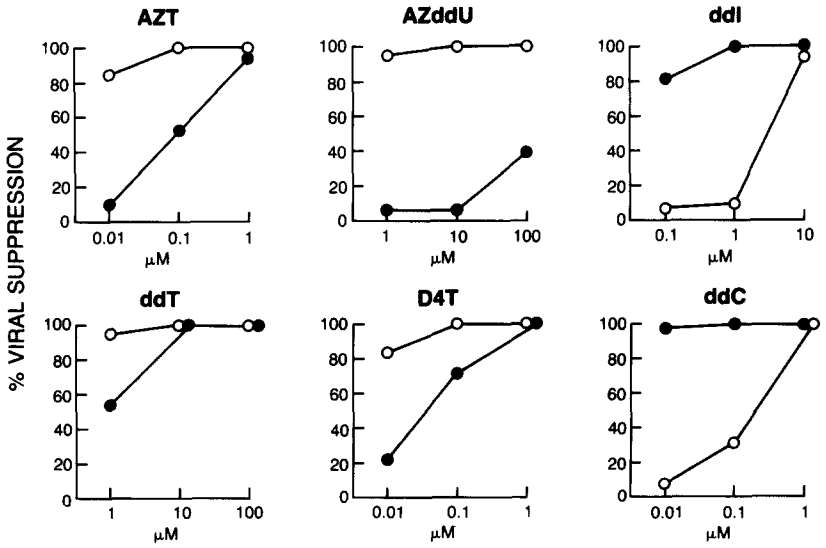

FIGURE 6. Same as Fig. 5, except that the lymphocytotropic strain $HTLV\text{-}III_B$ (1,000 viral particles/cell) was used. Viral replication was assessed by HIV-p24 gag production after 21 d of culture. Control gag production (without drugs) was as follows: M/M not treated with GM-CSF, 2,150 pg/ml; GM-CSF-treated M/M, 21,600 pg/ml. Results shown here represent a typical experiment.

III_{Ba-L} activity in fresh M/M in the presence of GM-CSF (Fig. 5 *B*). Comparable results were obtained using the other HIV-1 strain, HTLV-III_B (Fig. 6). Our data do not distinguish whether this represented unchanged anti-HIV activity in the presence of an increased efficiency of viral replication, or alternatively, an actual reduction of anti-HIV activity. In either case, the results indicate that GM-CSF seems to enhance the anti-HIV effect of AZT and its congeners in M/M, while the effect of several other dideoxynucleosides is unchanged or possibly even reduced in M/M cultured with this cytokine.

To determine whether the enhanced antiviral activity of AZT and its congeners by GM-CSF might be due to an increased cell toxicity, we assessed this parameter by two different methods. In the absence of GM-CSF, M/M failed to show any drug-related toxicity, as assessed by trypan blue exclusion, at concentrations up to 100 μM AZT, 500 μM ddT, 10 μM D4T, or 100 μM AZddU (data not shown). Also, no inhibition of phagocytic activity was seen at the same concentrations. In the presence of GM-CSF, phagocytosis was substantially unaffected by drugs (Table II). However, high concentrations of AZT and AZddU did lower the number of M/M exposed to GM-CSF in a dose-dependent fashion (Table II); in the case of AZT, a 50% reduction of viable M/M was obtained at 10 μM. On the other hand, 1 μM AZT was devoid of cytotoxicity in monocytes exposed to GM-CSF, and this concentration is >100 times the ED_{50} described earlier under these conditions. Similarly, ddT and D4T did not substantially affect cell viability at concentrations up to 500 and 10 μM, respectively. Thus, while GM-CSF does have an effect on the toxicity of AZT and analogues at the same time as it increases their antiviral activity, >95% HIV suppression by AZT analogues in GM-CSF-treated M/M is obtained at concentrations 10–500 times (depending on different AZT analogues) lower than those necessary to induce such viral inhibition in HIV-exposed T lymphocytes; moreover,

TABLE II

Toxicity of Dideoxynucleosides in M/M Exposed to GM/CSF

Drug	Concentration	Total viable cells		Phagocytic activity (percent positive cells)
		$\times 10^4$/ml	Percent of control	
	μM			
No drug	-	5.2	-	92
AZT	1	6.1	>100	90
	10	2.6	50	89
	100	2.4	46	88
AZddU	10	5.7	>100	98
	100	3.7	71	95
	1,000	3.1	60	94
ddT	10	6.4	>100	95
	100	5.4	>100	93
	500	6.3	>100	96
D4T	1	6.4	>100	96
	10	4.6	89	93

10^5 fresh elutriated M/M exposed to 100 U/ml GM-CSF were cultivated for 14 d in 2 ml of complete medium in the presence of different concentrations of ddN. Viability was measured by trypan blue exclusion method, and phagocytosis was evaluated after a 1-h incubation with 0.8 μ latex beads; viable cells ingesting at least two beads were considered positive.

in the case of AZT, little or no toxicity is observed at drug concentrations typically attained in vivo (1–3 μM).

To evaluate some possible mechanisms responsible for the interaction of GM-CSF and AZT congeners in fresh M/M cultivated for 5 d, we studied the metabolism of AZT and D4T in M/M with or without GM-CSF. The results are summarized in Fig. 7 and in Table III. In the absence of GM-CSF, M/M had low levels of intracellular AZT-5'-triphosphate (AZTTP). As can be seen by the high AZT-5'-monophosphate/AZT-5'-diphosphate (AZTMP/AZTDP) ratio in Fig. 7 *A*, GM-CSF does not eliminate the relative block at the level of thymidylate kinase that is characteristic for AZT metabolism. Exposure of the M/M to GM-CSF dramatically increased the intracellular levels of both parent AZT and its mono-, di-, and triphosphate anabolites. In particular, AZTTP level increased >15-fold. These effects were not seen in H9 T cell line exposed to GM-CSF. In part, the increase in AZTTP in M/M exposed to GM-CSF may be due to an increase in the cell entry of AZT, as evidenced by the higher intracellular levels of AZT as parent compound. Also, GM-CSF may have an effect on the level of intracellular thymidine kinase. The activity of this enzyme, which catalyzes the initial phosphorylation of AZT and related congeners, and thymidine as well, is very low in M/M when compared with T cells (8, 31). M/M exposed to GM-CSF had approximately a twofold increase in thymidine kinase compared with control M/M cultured without GM-CSF (data not shown). A similar increase in phosphorylation was obtained with D4T: active metabolites of D4T were barely detectable in M/M unexposed to GM-CSF, whereas large amounts of both

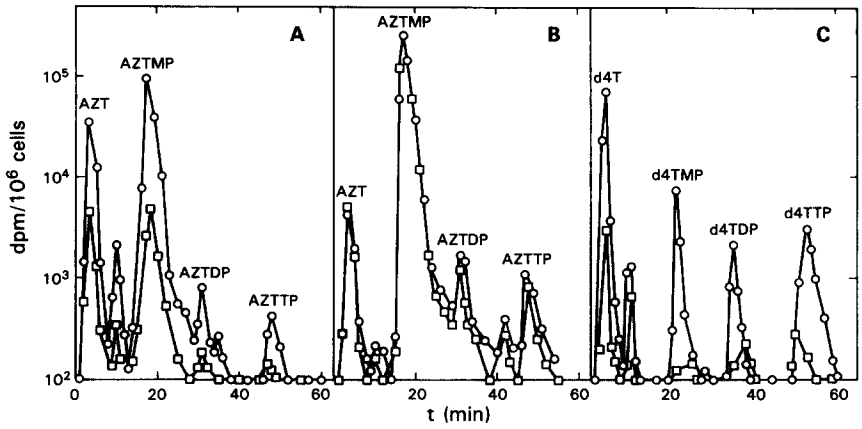

FIGURE 7. Typical ion exchange (Partisil-10 SAX) HPLC elution profile of 60% methanolic extracts of elutriated M/M (*A*) or H9 T cells (*B*) incubated for 24 h with 10 μM [^{3}H]AZT (sp act 3 Ci/mmol) and of elutriated M/M incubated for 24 h with 10 μM [^{3}H]-2'3'-dideoxy-2'3'-dehydrothymidine (D4T, sp act 10 Ci/mmol) (*C*). 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 15 min isocratic elution with 0.6 M ammonium phosphate, pH 3.8. Sample volume: 200 μl. 1-min fractions were collected, and data are expressed as dpm/10^6 cells. Note that the ordinate utilizes a logarithmic rather than a linear scale because of the marked accumulation of AZT at the 5'-monophosphate level and the resulting disparity in peak heights between those for AZTMP and for AZTTP. (□) Control (no GM-CSF); (○) GM-CSF treated cells.

TABLE III

Intracellular Phosphorylation of AZT and D4T to Their Active Moieties; Comparisons to Levels of Endogenous Competing dTTP Pools in M/M and H9 T Cells Exposed to GM-CSF

Cell type	AZT	AZTMP	AZTDP	AZTTP	dTTP	AZTTP/dTTP
			pmol/10^6cells			
M/M	1.25	1.40	<0.015	<0.015	1.26	<0.012
M/M + GM-CSF	11.16	35.84	0.135	0.155	1.9	0.082
H9	1.29	78.59	0.4	0.42	27.9	0.015
H9 + GM-CSF	1.69	86.66	0.35	0.31	-	-
	D4T	D4TMP	D4TDP	D4TTP	dTTP	D4TTP/dTTP
			pmol/10^6 cells			
M/M	0.08	<0.015	<0.015	<0.015	1.26	<0.012
M/M + GM-CSF	4.42	0.45	0.16	0.49	1.9	0.258
H9	1.96	0.115	0.045	0.19	27.9	0.007

For phosphorylation studies, 5×10^6 M/M or H9 were cultivated for 5 d in 4 ml of complete medium in the presence or absence of GM-CSF 100 U/ml. After 5 d, cells were exposed to 10 μM [^{3}H]AZT, or 10 μM [^{3}H]D4T. After 24 h, cells were harvested (M/M after gentle scraping to detach adherent cells), counted, pelleted, and immediately frozen. Extraction was performed as described (see text). For endogenous dTTP pools, 2×10^7 cells were cultivated for 5 d with/without GM-CSF 100 U/ml, then harvested, counted, pelleted, and immediately frozen. Endogenous dTTP pools were estimated as described (see text).

D4T and its mono-, di-, and triphosphate moieties were detected in GM-CSF-exposed M/M. Indeed, the levels in such cells were comparable with those seen in H9 T cells.

We also studied the endogenous 2'-deoxythymidine-5'-triphosphate (dTTP) pool, which competes with AZTTP and related congeners at the level of viral RT. We found that the concentration of dTTP was very low in this population of M/M, as previously reported (8) for M/M tested under different conditions, and dTTP increased less than twofold in M/M exposed to GM-CSF for 5–7 d (Table III). In the current work, we observed that elutriated M/M cultured for 5 d in media alone have a very low level of endogenous dNTPs compared with the levels obtained in other M/M populations (8). Thus, GM-CSF-exposed M/M have a substantially higher ratio of AZTTP/dTTP than control M/M, a result that might explain the increased activity of AZT and related congeners in such cells.

Discussion

In this report, we have found that GM-CSF activates the replication of HIV-1 in fresh M/M cultured for 5 d, yet at the same time it potentiates the anti-HIV activity of AZT and related congeners in fresh M/M. Furthermore, we show that this effect on AZT may be accounted for by a preferential enhancement of AZT cell entry and subsequent phosphorylation induced by GM-CSF. Similar considerations apply to other dideoxy analogues of thymidine.

We wish to stress that any specific clinical or therapeutic inference from our data should be made only with great caution at this time. Nevertheless, the infection of cells belonging to the M/M lineage is increasingly recognized as an important event in the pathogenesis of AIDS (2, 5, 32). M/M are currently believed to be one of the earliest cells infected after exposure to HIV (33), and may play an important role in spreading HIV throughout the immune system (34). In addition, monocyte-

derived cells appear to be the primary target for HIV infection of the nervous system (2, 5, 35). Thus, factors that influence the degree of replication of HIV in M/M may influence the course of HIV infection in patients. While certain strains of HIV have been reported to replicate in peripheral blood monocytes in the absence of exogenous stimulation(2, 8, 32), we show here that stimulation of these cells by GM-CSF markedly enhances their capacity to permit replication of HIV. Indeed, even the lymphocytotropic strain of HIV-1, HTLV-III_B, which in our hands does not replicate well in unstimulated M/M, replicates quite efficiently in M/M exposed to GM-CSF. It is conceivable that in our system, GM-CSF stimulates the production of host-cell transcriptional factors that in turn can potentiate viral transcription or posttranscriptional events (36).

It is of note that another marrow growth factor, macrophage-CSF (M-CSF or CSF-1) has been shown by Gendelman et al. (37) to enhance HIV production by M/M. In the case of CSF-1-stimulated M/M, however, most of the HIV production was reported to be sequestered in vacuoles, while with GM-CSF, abundant virus was released into the media. Even though we failed to find evidence of an increase in p24 released into the media by disrupting GM-CSF-exposed M/M using three cycles of freeze-thawing, this would not completely exclude the possibility that GM-CSF induced a limited form of virus sequestration in intracytoplasmic vacuoles; to specifically address this point, one would have to examine GM-CSF-exposed HIV-infected M/M by EM.

It is of interest that GM-CSF treatment of target M/M potently enhances the replication of the lymphocytotropic strain HTLV-III_B in these cells; in this context GM-CSF has a net effect of converting a lymphocytotropic virus to a monocytotropic virus (Fig. 4). Thus, the available data from this and other studies suggest that GM-CSF is, if anything, an even more potent stimulus for HIV replication in M/M than CSF-1 (37, 38). GM-CSF is produced by a number of cells in vivo (39–45). As one such cell is the T lymphocyte (39, 42, 43), it is possible that GM-CSF might play a central role in the progression of HIV infection to AIDS. Antigenic stimulation of T cells may lead to GM-CSF production, which may then lead to enhanced HIV replication in macrophages and, in turn, spread of virus to more T cells.

At the same time, GM-CSF markedly enhances the anti-HIV effect of AZT and closely related drugs in M/M. It appears to do so by substantially increasing intracellular AZTTP levels, while exerting only a slight effect on the levels of competing endogenous dTTP. To some degree, the enhanced phosphorylation of AZT may be due to a GM-CSF-induced increase in thymidine kinase (which not only catalyzes the initial phosphorylation of thymidine and related ddN analogues, but also is reported to act on AZddU [30]). However, this does not appear to be the only mechanism responsible. M/M stimulated with GM-CSF and exposed to AZT have substantially higher levels of intracellular AZT as unphosphorylated compound than unstimulated M/M, suggesting that GM-CSF may also selectively enhance the cell entry of AZT and related drugs into M/M. It is worthwhile to point out that AZT and its normal counterpart, 2′-deoxythymidine, very likely permeate the cell membrane of lymphocytes and erythrocytes via different mechanisms (46); thus, lymphokines may have differential effects on the entry of different nucleosides. Also, in the case of the physiologic 2′-deoxynucleoside-5′-triphosphate, some of the endogenous thymidine pools may depend heavily on de novo synthesis from 2′-deoxyuridine-5′-mono-

phosphate (dUMP), and be regulated by feedback mechanisms. The physiologic 2'-deoxynucleotide may therefore not be affected in precisely the same way as those arising from ddN, by changes in membrane transport or diffusion. Additional studies will be required to detail the mechanism(s) responsible for the effect of GM-CSF on the phosphorylation of AZT and related analogues, and the mechanisms responsible for the reduced antiviral activity of ddN not related to AZT in GM-CSF-exposed M/M.

We are presently in the process of evaluating how exposure of M/M to GM-CSF affects the metabolism of another dideoxypyrimidine, ddC, and a dideoxypurine, ddA, in order to delineate the basis for the differential effects observed on antiviral activity. Preliminary results indicate that by contrast to the >10-fold enhancement of AZTTP induced by GM-CSF, there is only a twofold enhancement of ddCTP. The competing physiologic nucleotide, 2'-deoxycytidine-5'-triphosphate (dCTP), however, was below the limit of detection of our assay in both of these cultured populations of M/M, and for this reason, we could not calculate the effect of GM-CSF on the ddCTP/dCTP ratio. Also, while ddATP and dATP were measurable in M/M exposed to GM-CSF, they were below the limit of detection in the media-control (unstimulated) M/M. Thus, while these preliminary results suggest that the increase in ddCTP induced by GM-CSF was substantially less than the increase of AZTTP, and that the increase of ddATP was likely coupled with a substantial increase in the competing dATP, additional experiments will be needed to fully characterize the effects of GM-CSF on the intracellular biochemistry of these dideoxynucleosides. Nevertheless, we can speculate that the limited net activation of metabolism of such nonthymidine ddN is probably not sufficient to overcome the dramatic increase in viral yield induced by GM-CSF stimulation.

GM-CSF is currently being studied as a supplementary drug for the treatment of AIDS because of the possibility that it may counteract the marrow suppression induced by HIV infection or by AZT administration. The results reported here suggest that one may wish to closely monitor patients when using GM-CSF in the setting of HIV-infection since it might enhance viral replication. However, as also shown here, the simultaneous administration of GM-CSF with AZT, D4T, or related analogues may induce a potent antiviral effect in monocytes. Could such a combination also increase AZT toxicity in vivo in marrow blood-forming cells? The answer is not known; however, a recent study suggested that in contradistinction to our results obtained in M/M, GM-CSF might reduce the phosphorylation of AZT in bone marrow cells while increasing the levels of thymidine triphosphate (47). It is thus conceivable that GM-CSF may act to increase the overall therapeutic index of AZT and related thymidine analogues. The simultaneous administration of these two agents, therefore, may be worth exploring in patients with severe HIV infection.

Summary

We have investigated the influence of granulocyte-macrophage CSF (GM-CSF) on the replication of HIV-1 in cells of monocyte/macrophage (M/M) lineage, and its effect on the anti-HIV activity of several 2'3'-dideoxynucleoside congeners of thymidine in these cells in vitro. We found that replication of both HTLV-III$_{Ba-L}$ (a monocytotropic strain of HIV-1) and HTLV-III$_B$ (a lymphocytotropic strain) is markedly enhanced in M/M, but not in lymphocytes exposed to GM-CSF in cul-

ture. Moreover, GM-CSF reduced the dose of HIV required to obtain productive infection in M/M. Even in the face of this increased infection, GM-CSF also enhanced the net anti-HIV activity of 3′-azido-2′3′-dideoxythymidine (AZT) and several related congeners: 2′3′-dideoxythymidine (ddT), 2′3′-dideoxy-2′3′-didehydrothymidine (D4T), and 3′-azido-2′3′-dideoxyuridine (AZddU). Inhibition of viral replication in GM-CSF-exposed M/M was achieved with concentrations of AZT and related drugs, which were 10–100 times lower than those inhibitory for HIV-1 in monocytes in the absence of GM-CSF. Other dideoxynucleosides not related to AZT showed unchanged or decreased anti-HIV activity in GM-CSF-exposed M/M. To investigate the possible biochemical basis for these effects, we evaluated the metabolism of several drugs in M/M exposed to GM-CSF. We observed in these cells markedly increased levels of both parent and mono-, di-, and triphosphate anabolites of AZT and D4T compared with M/M not exposed to GM-CSF. By contrast, only limited increases of endogenous competing 2′-deoxynucleoside-5′-triphosphate pools were observed after GM-CSF exposure. Thus, the ratio of AZT-5′-triphosphate/2′-deoxythymidine-5′-triphosphate and 2′3′-dideoxy-2′3′-didehydrothymidine-5′-triphosphate/2′-deoxythymidine-5′-triphosphate is several-fold higher in GM-CSF-exposed M/M, and this may account for the enhanced activity of such drugs in these cells.

Taken together, these findings suggest that GM-CSF increases HIV-1 replication in M/M, while at the same time enhancing the anti-HIV activity of AZT and related congeners in these cells. These results may have implications in exploring new therapeutic strategies in patients with severe HIV infection.

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