Effects of Bone Marrow Stimulatory Cytokines on Human Immunodeficiency Virus Replication and the Antiviral Activity of Dideoxynucleosides in Cultures of Monocyte/Macrophages


Cells of the monocyte lineage are important targets for the replication of human immunodeficiency virus (HIV). Our group and others have previously shown that granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates HIV replication in monocyte/macrophages, but that it also enhances the anti-HIV activity of 2',3'-dideoxy-3'-azidothymidine (AZT). In the present study, we have explored the effects of other bone marrow stimulatory cytokines on the replication of HIV and on the anti-HIV activity of certain dideoxynucleosides in human peripheral blood monocyte/macrophages (M/M). Like GM-CSF, macrophage CSF (M-CSF) enhanced HIV replication in M/M. In contrast, granulocyte CSF (G-CSF) and erythropoietin (Epo) had no such effects. The anti-HIV activity of zidovudine (AZT) was increased in M/M exposed to GM-CSF. In contrast, the anti-HIV activity of AZT was unchanged in M/M exposed to M-CSF, and the activities of 2',3'-dideoxycytidine (ddC) and 2',3'-dideoxyinosine (ddI) were unchanged or slightly diminished in M/M stimulated with GM-CSF or M-CSF. These differential activities of AZT and ddC were paralleled by differential effects of the cytokines on the anabolism of these drugs to their active 5'-triphosphate moieties. GM-CSF increased the levels of AZT-5'-triphosphate (at least in part through an increase in thymidine kinase activity) and overall induced an increase in the ratio of AZT-5'-triphosphate/thymidine-5'-triphosphate. In contrast, M-CSF-induced increases in AZT-5'-triphosphate were roughly matched by increases in thymidine-5'-triphosphate. Also, GM-CSF- or M-CSF-induced increases in the levels of ddC-5'-triphosphate were associated with parallel increases in the levels of deoxyxycytidine-5'-triphosphate (the physiologic nucleoside that competes at the level of reverse transcriptase), so that there was relatively little net change in the ddC-5'-triphosphate/deoxyxycytidine-5'-triphosphate ratio. Thus, bone marrow stimulatory cytokines may have a variety of effects on HIV replication and on the activity and metabolism of dideoxynucleosides in M/M.

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MATERIALS AND METHODS

Cells. M/M-enriched peripheral blood mononuclear cells were obtained from healthy, HIV-negative donors using a cell separator (Fenwal C3000; Travenol Inc, Deerfield, IL). M/M were then purified by countercurrent centrifugal elutriation, as described. Cells obtained by elutriation consisted of at least 95% M/M, as

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evaluated by non-specific esterase technique and by morphology (Giems staining). M/M were also greater than 93% CD11b- and CD36-positive (OKM1 and OKM5, respectively; Ortho Diagnostic System, Westwood, MA). Other characteristics of these cells have been described elsewhere.28

Cytokines. Human recombinant GM-CSF for most experiments was obtained from Sandzoe Research Institute (East Hanover, NJ). This GM-CSF preparation contains 5.4 x 10⁶ chronic myelogenous leukemia (CML) units per milligram of glycoprotein, as measured in a rapid CML proliferation assay.29 Some experiments were also repeated using GM-CSF from Amgen Biologicals (Thousand Oaks, CA). Recombinant M-CSF was provided by Genetics Institute (Cambridge Park, MA), and contains 2 x 10⁷ U/mg of protein, as evaluated in a murine bone marrow colony assay.30 Some experiments were also repeated using recombinant M-CSF produced by Cetus Corp (Emeryville, CA) and purchased through Cellular Products (Buffalo, NY). In particular, studies of AZT and ddC metabolism, of endogenous nucleotide pools, and of kinases were performed using both sources of GM-CSF and M-CSF; no consistent differences were noted between the various preparations. Recombinant Epo was provided by Genetics Institute and contains 2.6 x 10⁵ U/mg protein, as measured in a thymidine uptake assay using spleen cells from phenylhydrazine-treated mice.31 Recombinant G-CSF (Amgen Biologicals) used in these experiments had a specific activity of 1 x 10⁷ U/mg protein, as measured in human bone marrow-derived cells as previously described.32 Cytokines were reconstituted in distilled water and kept at 4°C until used. The cytokines were used at concentrations observed to stimulate bone marrow progenitor cells in vitro.

Virus. HTLV-IIIb41, a monocytotropic strain of HIV-1 (a kind gift of Drs S. Gartner, M. Popovic, and R. Gallo, National Cancer Institute, Bethesda, MD), was used in these experiments. This strain will be referred to hereafter as HIV-IIIb. Supernatants of HIV-infected cultures of human peripheral blood M/M were used as source of virus; they were filtered and stored in liquid nitrogen before use. The infectivity of lots of HIV-IIIb was determined in cultures of primary M/M. Details of viral titration and other characteristics of HIV-IIIb have been described elsewhere.28

Drugs. 3'-Azido-2',3'-dideoxythymidine (AZT, zidovudine; Wellcome Research Laboratories, Research Triangle Park, NC), ddC, and ddl (Pharmacia Fine Chemicals, Piscataway, NJ) were obtained from Moravek Biochemicals Inc (Brea, CA). ddC, and ddl (Pharmacia Fine Chemicals, Piscataway, NJ) were obtained from Moravek Biochemicals Inc (Brea, CA). ddC, and ddl were obtained with 500 to 1,000 U/mL M-CSF in 4 mL of complete medium. Control M/M were cultivated for 5 days in 4 mL of complete medium in 12-well plates (Costar) in the presence or absence of 100 U/mL GM-CSF or 500 to 1,000 U/mL M-CSF. After 5 days, M/M were exposed to 10 μmol/L [3H]dA (10 pCi/mL) or 10 μmol/L [3H]ddA (10 μCi/mL) (a prodrug rapidly converted to ddl by the intracellular and extracellular enzyme adenosine deaminase). After 24 hours, M/M were harvested by gentle scraping, counted, and repeatedly washed to remove cell-free radiolabeled material. Cell pellets were then extracted with 500 μL of 60% methanol (vol/vol), heated at 95°C for 1.5 minutes, and clarified by centrifugation at 12,000g for 6 minutes. Finally, 200 μL aliquots were loaded onto a radially compressed column of Whatman Partisil (Hillsboro, OR) 10 SAX analytical column and eluted with an ammonium phosphate gradient as previously described.25,26 Levels of 5'-monophosphate (MP), 5'-diphosphate (DP), and 5'-triphosphate (TP) metabolites were expressed as picomoles of drug metabolites/10⁶ cells.

Antiviral drug assay. The assessment of antiviral activity was performed with minor modifications of a previously published procedure.13 Briefly, elutriated M/M were cultured starting 5 days before infection (day −5) at a concentration of 10⁶ cells/mL in wells of a 48-well plate (Costar, Cambridge, MA) in 1 mL of RPMI 1640 medium (GIBCO Labs, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (Hyclone Labs, Logan, UT), 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (GIBCO) (hereafter referred to as complete medium). Also added to certain wells were 100 U/mL GM-CSF, 1,000 U/mL M-CSF, 500 U/mL G-CSF, or 2 U/mL of Epo as described. At day 0, M/M were exposed to different concentrations of AZT, ddC, or ddl. Twenty minutes later, the cells were challenged with 500 minimum infectious doses (MID)/well of HIV-IIIb. Titration to determine viral infectivity was performed in a primary M/M system; the MID was defined as the minimum amount of virus that induced viral replication in at least two of four M/M cultures. On day 2, M/M were extensively washed to remove excess virus, and then cultivated as before, with cytokines and drugs, at 37°C in a humidified atmosphere supplemented with 5% CO₂. Unless stated differently, cytokines were kept continuously in culture from day −5, and drugs were kept continuously in culture from day 0. Cells were washed and fed every 5 days. Starting on day 9, viral production was assessed at established time points by measurement of HIV-p24 gag production in the supernatants of infected cultures using a commercially available radioimmunoassay and/or an enzyme-linked immunosorbent assay as noted (DuPont Co, Wilmington, DE). In addition, syncytia formation was evaluated in infected cultures by visual inspection. In selected experiments, reverse transcriptase production was assessed as described.13

Toxicity. Toxicity from the combination of drugs and cytokines was evaluated by trypan blue exclusion and determination of viable cell number in mock-infected cultures. Toxicity was further assessed by a detection of 3H-thymidine (New England Nuclear, Boston, MA) incorporation in GM-CSF-treated M/M exposed to different concentrations of AZT, ddC, and ddl as previously described.13

Nucleoside kinase assay. Elutriated M/M, 5 x 10⁶, cultivated for 5 days with medium alone or with 100 U/mL GM-CSF, were harvested from the plates by gentle scraping followed by washing with cold RPMI. Pellets were resuspended in 100 to 200 μL of 200 μmol/L dithiothreitol (DTT) in 0.1 Tris-HCl and 15% glycerol (vol/vol) and stored at −70°C. 2'-Deoxycytidine kinase and 2'-deoxythymidine kinase activities were measured with minor modifications of a previously published procedure,33 using 10 μmol/L 2'-deoxycytidine or 475 μmol/L 2'-deoxythymidine as substrate in the presence of 500 μmol/L adenine triphosphate (ATP), 10 mmol/L MgCl₂, and 2 mmol/L DTT.

Drug metabolism. Anabolic phosphorylation of ddN in M/M exposed to cytokines was assessed using a previously published procedure.25,26 Briefly, 5 to 10 x 10⁶ well elutriated M/M were cultured for 5 days in 4 mL of complete medium in 12-well plates (Costar) in the presence or absence of 100 U/mL GM-CSF or 500 to 1,000 U/mL M-CSF. After 5 days, M/M were exposed to 10 μmol/L [3H]AZT (10 μCi/mL), 0.5 μmol/L [3H]ddC (8 μCi/mL), or 10 μmol/L [3H]ddA (10 μCi/mL) (a prodrug rapidly converted to ddl by the intracellular and extracellular enzyme adenosine deaminase). After 24 hours, M/M were harvested by gentle scraping, counted, and repeatedly washed to remove cell-free radiolabeled material. Cell pellets were then extracted with 500 μL of 60% methanol (vol/vol), heated at 95°C for 1.5 minutes, and clarified by centrifugation at 12,000g for 6 minutes. Finally, 200 μL aliquots were loaded onto a radially compressed column of Whatman Partisil (Hillsboro, OR) 10 SAX analytical column and eluted with an ammonium phosphate gradient as previously described.25,26 Levels of 5'-monophosphate (MP), 5'-diphosphate (DP), and 5'-triphosphate (TP) metabolites were expressed as picomoles of drug metabolites/10⁶ cells.

Endogenous nucleotide pools. For these experiments, 5 x 10⁷ elutriated M/M were exposed for 5 days to 100 U/mL GM-CSF or 500 to 1,000 U/mL M-CSF in 4 mL of complete medium. Control M/M were cultured without cytokines. The cells were then collected by gentle scraping, counted, and washed twice in phosphate-buffered saline at 4°C. The pellets were immediately frozen in dry ice. Deoxyribonucleoside triphosphates were then extracted with 60% methanol. The extracts were then heated for 5 minutes at 95°C and centrifuged. The supernatant fractions were removed and assayed to determine the levels of endogenous deoxythymidine-5'-triphosphate (dTTP) and deoxythymidine-5'-triphosphate (dCTP) using the method of Sherman and Frye.35

Statistics. The statistical differences in HIV replication between control M/M and M/M exposed to GM-CSF or M-CSF were assessed using the two-tailed rank sum test. Statistical analysis of the difference of viral suppression by AZT in control M/M versus GM-CSF- or M-CSF-exposed M/M has been performed using the two-tailed signed rank test.
HIV REPLICATION IN MONOCYTE/MACROPHAGES

RESULTS

As a prelude to the study of the effects of various cytokines on the anti-HIV activity of dideoxynucleosides in M/M, we first assessed the effect of these cytokines on HIV replication in our system. In agreement with previously published results,12,13,16 we found that GM-CSF (100 U/mL) and M-CSF (1,000 U/mL) both induced a significant enhancement of HIV replication as compared with control M/M (P < .01 and P < .05 for GM-CSF and M-CSF, respectively) (Fig 1). Enhancement of HIV replication by these two cytokines was achieved in all experiments. The increase was more pronounced and seen earlier with GM-CSF, and with this cytokine, there was a 7- to 30-fold increase in peak p24 antigen production at the time points assayed as compared with untreated cultures. As might be expected, G-CSF (at concentrations up to 500 U/mL) had no effect on HIV replication. Also, Epo (at concentrations up to 2 U/mL) induced no consistent effect on HIV replication (although there was a slight increase in occasional experiments). Syncytia formation was observed in HIV-infected cultures containing GM-CSF or M-CSF, starting on day 9 and persisting for at least 3 weeks. In contrast, syncytia were not observed in control HIV-infected cultures or those exposed to G-CSF or Epo.

With this background, we then assessed the effect of these cytokines on the anti-HIV activity of various dideoxynucleosides. As shown in Fig 2, 35% inhibition of de novo HIV infection was observed with 0.01 μmol/L AZT in control M/M (not exposed to cytokines), while approximately 75% inhibition was obtained with 0.1 μmol/L AZT. In agreement with our previous report,13 we observed that GM-CSF substantially enhanced the antiviral activity of AZT. For example, in the presence of 100 U/mL GM-CSF, the viral suppression obtained with 0.01 μmol/L of AZT increased from a mean of 35% to greater than 98%. In contrast, the percent viral suppression was nearly unchanged in the presence of 1,000 U of M-CSF, 500 U/mL of G-CSF, or 2 U of Epo (Table 1 and Fig 2). (It should be noted that the net viral production in the presence of a given concentration of AZT was substantially greater in M-CSF-exposed cultures than control cultures. However, the viral production in the absence of AZT was also higher in M-CSF-exposed cultures, so there was no change in the percent inhibition.)

Assessment of reverse transcriptase activity in the cultures paralleled the results attained by measurement of HIV p24 antigen. Similar results were attained when syncytia formation was examined in GM-CSF- and M-CSF-exposed M/M (data not shown). Also, while the data shown in Table 1 and Fig 2 were obtained with GM-CSF from Sandoz Research Institute and with M-CSF from Genetics Institute, similar results were obtained with GM-CSF from Amgen Biologicals and with M-CSF manufactured by Cetus Corp (data not shown). Finally, no clear evidence of toxicity, as assessed by trypan blue exclusion test and, in the case of GM-CSF–treated M/M, by thymidine incorporation, was found in either control or cytokine-treated M/M at concentrations of AZT up to 50 to 100 times greater than those able to induce greater than 95% inhibition of viral replication (data not shown).

In additional experiments, we examined lower doses of these cytokines. In the case of GM-CSF, enhancement of HIV replication (in the absence of AZT) decreased with concentrations below 100 U/mL; at 1 U/mL, little or no
enhancement was observed. Likewise, no substantial effect of GM-CSF on AZT activity was observed at 1 U/mL. In the case of M-CSF, the effects on HIV replication and AZT activity likewise were less pronounced at concentrations below 1,000 U/mL (data not shown).

We next assessed the antiviral activity of ddC and ddI in M/M exposed to various marrow-stimulatory cytokines. As shown in Fig 3, the activity of ddC was essentially unchanged in M/M exposed to Epo. However, the percent viral suppression induced by a given concentration of ddC appeared to be somewhat reduced in the presence of 100 U/mL GM-CSF or 1,000 U/mL M-CSF (Fig 3 and Table 2). Assessment of the antiviral activity of anti-HIV agents is highly dependent on the viral load, and it is difficult to separate an actual decrease in anti-HIV activity from an apparent decrease caused by a cytokine-induced increase in viral load. However, it should be noted that even in the presence of GM-CSF or M-CSF, 0.1 μmol/L ddC (a concentration which can be obtained in the plasma of patients) was able to induce greater than 99% inhibition of viral replication in M/M. Toxicity induced by ddC in cytokine-exposed M/M was absent at concentrations of up to 1 μmol/L of ddC.

The results obtained with ddI were similar to those seen with ddC (Fig 4, Table 2). This drug showed potent anti-HIV activity in control M/M and in Epo-treated M/M; an average of 65% suppression of viral replication was achieved with 0.1 μmol/L ddI 21 days after viral challenge, and greater than 95% suppression was attained with 1 μmol/L of ddI (Fig 4). As with ddC, there appeared to be a slight decrease in the percent inhibition of viral replication induced by ddI in M/M exposed to GM-CSF or M-CSF at any time point in which viral replication was assessed (Fig 4, Table 2). Again, this apparent decrease in anti-HIV activity of ddI may have simply resulted from the cytokine-induced increased viral load.

We next tried to assess the biochemical mechanism for the differential effects of GM-CSF and M-CSF on the anti-HIV activity of AZT and ddC. (We also attempted to assess the phosphorylation of ddA/ddI, but the assay used was not sufficiently sensitive to reliably measure the levels of ddA-5'-triphosphate in the M/M.) We had previously shown that levels of AZT-5'-triphosphate (AZTTTP) are substantially higher in GM-CSF-stimulated M/M than in control M/M.13 In the present experiments, we also observed a consistent increase in the level of AZT-5'-triphosphate in M/M exposed to GM-CSF; on average, AZTTTP levels were 3.8 times higher in GM-CSF-exposed M/M than in control M/M (Table 3). In addition, as we had previously observed, GM-CSF-stimulated M/M had somewhat increased levels of thymidine-5'-triphosphate (dTTP), the physiologic nucleotide that competes with AZTTTP at the levels of reverse transcriptase. However, the increase in dTTP levels did not compensate for the increase in AZTTTP, so that GM-CSF treatment resulted in a nearly threefold increase in the ratio of AZTTTP/dTTP. Previous studies have suggested that this ratio is an important determinant of anti-HIV activity in various cell types.13 It should be noted that there was more experiment-to-experiment heterogeneity in the M/M populations. M-CSF treatment
Table 2. HIV p24 gag Production and Viral Suppression by Different Concentrations of ddC or ddl in M/M Exposed to Different Cytokines

<table>
<thead>
<tr>
<th>Days After Infection</th>
<th>Drug (µmol/L)</th>
<th>No Cytokines</th>
<th>GM-CSF</th>
<th>M-CSF</th>
<th>Epo</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td></td>
<td>565</td>
<td>32,020</td>
<td>16,050</td>
<td>467</td>
</tr>
<tr>
<td>ddC 0.001</td>
<td>&lt; 312 (&gt; 44.7)</td>
<td>30,790 (3.5)</td>
<td>11,070 (31)</td>
<td>&lt; 312 (&gt; 32)</td>
<td></td>
</tr>
<tr>
<td>ddC 0.01</td>
<td>&lt; 312 (&gt; 44.7)</td>
<td>7,044 (78)</td>
<td>3,691 (77)</td>
<td>&lt; 312 (&gt; 32)</td>
<td></td>
</tr>
<tr>
<td>ddC 0.1</td>
<td>&lt; 312 (&gt; 44.7)</td>
<td>&lt; 312 (&gt; 99)</td>
<td>&lt; 312 (&gt; 98)</td>
<td>&lt; 312 (&gt; 32)</td>
<td></td>
</tr>
<tr>
<td>ddC 1</td>
<td>&lt; 312 (&gt; 44.7)</td>
<td>&lt; 312 (&gt; 99)</td>
<td>&lt; 312 (&gt; 98)</td>
<td>&lt; 312 (&gt; 32)</td>
<td></td>
</tr>
<tr>
<td>ddl 0.01</td>
<td>320 (43.4)</td>
<td>35,550 (0)</td>
<td>18,240 (0)</td>
<td>770 (0)</td>
<td></td>
</tr>
<tr>
<td>ddl 0.1</td>
<td>&lt; 312 (&gt; 44.7)</td>
<td>10,570 (67)</td>
<td>15,175 (5.5)</td>
<td>315 (31)</td>
<td></td>
</tr>
<tr>
<td>ddl 1</td>
<td>&lt; 312 (&gt; 44.7)</td>
<td>4,510 (85.9)</td>
<td>7,550 (53)</td>
<td>&lt; 312 (&gt; 32)</td>
<td></td>
</tr>
<tr>
<td>ddl 10</td>
<td>&lt; 312 (&gt; 44.7)</td>
<td>&lt; 312 (&gt; 99)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>2,450</td>
<td>250,400</td>
<td>58,625</td>
<td>4,262</td>
</tr>
<tr>
<td>ddC 0.001</td>
<td>735 (70)</td>
<td>230,360 (8)</td>
<td>36,106 (35)</td>
<td>1,619 (62)</td>
<td></td>
</tr>
<tr>
<td>ddC 0.01</td>
<td>&lt; 312 (&gt; 87.3)</td>
<td>62,600 (75)</td>
<td>11,130 (81)</td>
<td>&lt; 312 (&gt; 92.7)</td>
<td></td>
</tr>
<tr>
<td>ddC 0.1</td>
<td>&lt; 312 (&gt; 87.3)</td>
<td>350 (99.9)</td>
<td>&lt; 312 (100)</td>
<td>&lt; 312 (&gt; 92.7)</td>
<td></td>
</tr>
<tr>
<td>ddC 1</td>
<td>&lt; 312 (&gt; 87.3)</td>
<td>&lt; 312 (100)</td>
<td>&lt; 312 (100)</td>
<td>&lt; 312 (&gt; 92.7)</td>
<td></td>
</tr>
<tr>
<td>ddl 0.01</td>
<td>1,100 (55.1)</td>
<td>220,100 (12.1)</td>
<td>50,500 (13.9)</td>
<td>3,700 (13.2)</td>
<td></td>
</tr>
<tr>
<td>ddl 0.1</td>
<td>346 (85.9)</td>
<td>75,650 (69.8)</td>
<td>46,040 (21.5)</td>
<td>1,850 (56.6)</td>
<td></td>
</tr>
<tr>
<td>ddl 1</td>
<td>&lt; 312 (&gt; 87.3)</td>
<td>44,450 (82.3)</td>
<td>27,250 (53.5)</td>
<td>&lt; 312 (&gt; 92.7)</td>
<td></td>
</tr>
<tr>
<td>ddl 10</td>
<td>&lt; 312 (&gt; 87.3)</td>
<td>350 (99.9)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Elutriated M/M were exposed to various cytokines for 5 days and then challenged with HIV in the presence of different concentrations of ddC or ddl as described in Materials and Methods. Viral replication was assessed by HIV-p24 gag production; results shown are the values at days 9 and 13. In this representative experiment, viral replication in the presence of GM-CSF or M-CSF is substantially higher than that achieved in the experiment shown in Table 1. The percent suppression is shown in parentheses. Similar percentages of viral suppression by drugs were obtained in other experiments in which lower levels of viral replication were achieved.

Abbreviation: ND, not done.

of M/M also induced an increase in the levels of AZTTP to 2.3 times the control level (Table 3). However, M-CSF-treated M/M also had an increase in the level of dTTP, so that the AZTTP/dTTP ratio in M-CSF-treated M/M was slightly less than twofold higher than that in control M/M.

A somewhat different pattern was observed when the metabolism of ddC was studied. Both GM-CSF and M-CSF induced a twofold to threefold increase in the level of ddCTP in M/M (Table 3). However, both cytokines also induced a similar increase in the levels of dCTP (the physiologic nucleoside that competes with ddCTP at the level of reverse transcriptase). Thus, the ddCTP/dCTP ratio was less than twofold higher in GM-CSF– or M-CSF–treated M/M than in control M/M. In particular, the ddCTP/dCTP ratio in GM-CSF-stimulated M/M was almost identical to that of control M/M.

We had previously shown that the increased levels of AZTTP in GM-CSF–stimulated M/M were caused in part because of enhanced entry of AZT into the cells and in part because of enhanced phosphorylation to the 5′-triphosphate moiety. To extend this observation, we examined the levels of 2′-deoxythymidine kinase and 2′-deoxyuridine kinase, the enzymes that phosphorylate the initial phosphorylation of AZT and ddC, respectively, to their 5′-monophosphate moieties (Table 4). Both GM-CSF and M-CSF induced increases in the levels of 2′-deoxythymidine kinase and 2′-deoxyuridine kinase. Indeed, the levels of 2′-deoxyuridine kinase in GM-CSF– or M-CSF–stimulated M/M increased to those found in replicating H9 cells. In contrast, the levels of 2′-deoxythymidine kinase in cytokine-stimulated M/M remained well below the levels found in H9 cells. Thus, the increased levels of AZTTP and ddCTP in GM-CSF– and M-CSF–exposed M/M were in part the result of increased levels of the kinases involved in their initial phosphorylation to 5′-monophosphates.

**DISCUSSION**

Bone marrow suppression is a frequent complication in patients with HIV infection, either as a result of the underlying disease or certain therapeutic modalities.16,18,19,37-39
by the Food and Drug Administration of the United States produced by recombinant technology, and three of them, which the levels of endogenous cytokines may be elevated. Epo, GM-CSF, and G-CSF, have recently been approved for use in certain clinical conditions. M-CSF shows are the means (SEM) of three separate experiments. Epo, GM-CSF, and G-CSF have been observed to increase HIV replication in vitro. However, these effects must be considered in contemplating their activity of certain anti-HIV drugs in M/M, and these effects were in part paralleled by differences in AZT metabolism: GM-CSF exposure resulted in a consistent increase in the intracellular levels of AZTTP in M/M with a mean increase of almost fourfold, whereas M-CSF exposure induced a smaller increase (Table 3). Previous experiments in T cells and M/M have indicated that the ratio of dideoxynucleoside-5'-triphosphates to the physiologic dideoxynucleoside-5'-triphosphates is a more important determinant of the activity of these drugs than the absolute levels of the dideoxynucleoside-5'-triphosphates. While GM-CSF induced a small increase in the levels of dTTP, this was of a smaller magnitude than the increase in AZTTP, so that overall, this cytokine induced a 2.7-fold increase in the AZTTP/dTTP ratio.

While there was more variation in the drug metabolism in the M-CSF-stimulated cultures, this cytokine also appeared to yield a small net increase in the ratio of AZTTP/dTTP. However, it has been shown that the activity of antiviral drugs is highly dependent on the viral load. For M-CSF-stimulated M/M, a small increase in the ratio of AZTTP/dTTP appears to be roughly balanced by the increase in viral load. Similarly, for ddC, any increases in the ddCTP/dCTP ratios induced by GM-CSF or M-CSF are more balanced by the cytokine-induced increase in viral load, and the net effect is either unchanged anti-HIV activity or a slight decrease. In contrast, as noted above, the increase in AZTTP/dTTP ratios induced by GM-CSF would appear to! more than compensate for the cytokine-induced increase in viral load, and a net increase in anti-HIV activity is observed.

We had previously reported that the enhanced levels of AZTTP in GM-CSF-stimulated M/M are caused by both increased entry of AZT into the cells as well as increased phosphorylation to the 5'-triphosphate moiety. We now show further that the increase in AZTTP may be caused in part by increased levels of thymidine kinase in GM-CSF—

**Table 3. Intracellular Phosphorylation of AZT and ddC to Their Active Moieties: Comparison With Levels of Endogenous Competing dTTP and dCTP Pools in M/M Exposed to GM-CSF and M-CSF**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>AZTMP</th>
<th>AZTDP</th>
<th>AZTTP</th>
<th>dTTP</th>
<th>Ratio AZTTP/dTTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.18 (1.27)</td>
<td>0.31 (0.16)</td>
<td>0.20 (0.08)</td>
<td>2.65 (1.98)</td>
<td>0.074</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>191 (94)</td>
<td>1.25 (0.63)</td>
<td>0.76 (0.43)</td>
<td>3.91 (2.74)</td>
<td>0.20</td>
</tr>
<tr>
<td>M-CSF</td>
<td>78.9 (69.1)</td>
<td>0.59 (0.51)</td>
<td>0.46 (0.41)</td>
<td>3.50 (2.25)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

For phosphorylation studies, 5 to 10 × 10⁶ cells of elutriated M/M were cultured for 5 days in the presence or absence of GM-CSF (100 U/mL) or M-CSF (1,000 U/mL). At this point, the cells were exposed to 10 µmol/L [3H]AZT (10 µCi/mL) or 0.5 µmol/L [3H]ddC (8 µCi/mL). After 24 hours, the cells were harvested, counted, and washed. Cell pellets were then extracted and assayed as described in Materials and Methods. Results shown are the means (SEM) of four separate experiments. Endogenous dTTP and dCTP pools were determined as described in Materials and Methods. Results shown are the means (SEM) of three separate experiments.

Several bone marrow stimulatory cytokines have been produced by recombinant technology, and three of them, Epo, GM-CSF, and G-CSF, have recently been approved by the Food and Drug Administration of the United States for use in certain clinical conditions. As such, there is an interest in exploring their potential utility in enhancing bone marrow function in patients with HIV infection. However, as demonstrated in this study, these cytokines may have a variety of effects on HIV replication and on the activity of certain anti-HIV drugs in M/M, and these effects must be considered in contemplating their use in HIV-infected patients. Moreover, it is possible that these effects may be operative in certain patients in which the levels of endogenous cytokines may be elevated.

As has been previously reported, two cytokines that are stimulatory for M/M (GM-CSF and M-CSF) were observed to increase HIV replication in vitro. However, these two cytokines had differential effects on the in vitro anti-HIV activity of AZT: GM-CSF enhanced the anti-HIV activity of AZT in M/M, while M-CSF appeared to yield a small net increase in the ratio of AZTTP/dTTP. However, it has been shown that the activity of antiviral drugs is highly dependent on the viral load. For M-CSF-stimulated M/M, a small increase in the ratio of AZTTP/dTTP appears to be roughly balanced by the increase in viral load. Similarly, for ddC, any increases in the ddCTP/dCTP ratios induced by GM-CSF or M-CSF are more balanced by the cytokine-induced increase in viral load, and the net effect is either unchanged anti-HIV activity or a slight decrease. In contrast, as noted above, the increase in AZTTP/dTTP ratios induced by GM-CSF would appear to more than compensate for the cytokine-induced increase in viral load, and a net increase in anti-HIV activity is observed.

We had previously reported that the enhanced levels of AZTTP in GM-CSF-stimulated M/M are caused by both increased entry of AZT into the cells as well as increased phosphorylation to the 5'-triphosphate moiety. We now show further that the increase in AZTTP may be caused in part by increased levels of thymidine kinase in GM-CSF—

**Table 4. 2'-Deoxythymidine Kinase and 2'-Deoxycytidine Kinase in Extracts of Elutriated M/M Exposed to Cytokines or to Medium Alone**

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>2'-Deoxythymidine Kinase (pmol dTMP/mg protein/min)</th>
<th>2'-Deoxycytidine Kinase (pmol dCDP/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control M/M</td>
<td>10.5 ± 0.7</td>
<td>5.7 ± 2.8</td>
</tr>
<tr>
<td>M/M exposed to 100 U/mL GM-CSF</td>
<td>15.1 ± 2.9</td>
<td>10.9 ± 4.3</td>
</tr>
<tr>
<td>M/M exposed to 1,000 U/mL M-CSF</td>
<td>18.2 ± 2.2</td>
<td>14.8 ± 3.5</td>
</tr>
<tr>
<td>H9 cells</td>
<td>61.9 ± 25.1</td>
<td>11.0 ± 5.0</td>
</tr>
</tbody>
</table>

Activities of 2'-deoxythymidine kinase and 2'-deoxycytidine kinase were assessed in extracts of cells that had been cultured in media alone or media containing 100 U GM-CSF or 1,000 U M-CSF for 5 days. Results shown are the mean ± SEM of four separate experiments. Activities of 2'-deoxythymidine kinase and 2'-deoxycytidine kinase were assessed in extracts of cells that had been cultured in media alone or media containing 100 U GM-CSF or 1,000 U M-CSF for 5 days. Results shown are the mean ± SEM of four separate experiments. Activities of 2'-deoxythymidine kinase and 2'-deoxycytidine kinase were assessed in extracts of cells that had been cultured in media alone or media containing 100 U GM-CSF or 1,000 U M-CSF for 5 days. Results shown are the mean ± SEM of four separate experiments.

Abbreviations: dTMP, 2'-deoxythymidine-5'-monophosphate; dCMP, 2'-deoxycytidine-5'-monophosphate.
stimulated M/M. A similar enhancement of AZT phosphorylation and of thymidine kinase activity has recently been reported by Dhawan et al35 in a CD4+ monocytoid cell line (U937). These investigators also found that the degradation of AZTTP was somewhat slowed in GM-CSF-exposed U937 cells. Thus, a variety of mechanisms, including increased entry into cells, increased phosphorylation (caused in part by increased levels of thymidine kinase), and reduced degradation of the 5'-triphosphate, all may contribute to the increased levels of AZTTP in M/M or monocytoid cell lines.33,43

However, it should be noted that M-CSF exposure also induces increased levels of 2'-deoxycytidine kinase, and that both GM-CSF and M-CSF induce increases in 2'-deoxycytidine kinase (Table 4). Yet in these instances, increased kinase levels are not associated with an increased in drug activity. It should be recalled that in addition to catalyzing the initial phosphorylation of AZT and ddC, respectively, to their 5'-monophosphate moieties, these enzymes catalyze the initial step in the synthesis of dTTP and dCTP by the salvage pathway. dTTP and dCTP may also be produced by de novo synthesis, and their levels are regulated by a variety of complex factors. Thus, increased kinase levels may be associated with increased, decreased, or unchanged dideoxynucleoside-5'-triphosphate/dideoxynucleoside-5'-triphosphate ratios, and each combination of a cytokine and dideoxynucleoside has to be considered in its own right.

The clinical significance of these observations is not known. In a study conducted by our group, each of six evaluable patients who received a 2-week course of subcutaneous GM-CSF without AZT had increased serum HIV p24 antigen, suggesting (but not proving) that increased viral replication was occurring.21 Also, Kaplan et al44 have reported that in a trial of cytotoxic chemotherapy without dideoxynucleoside therapy for HIV-associated non-Hodgkin’s lymphoma, patients who received GM-CSF had higher serum HIV p24 antigen levels as compared with those who did not receive GM-CSF.44 On the other hand, in a trial of intravenously administered GM-CSF conducted by Groopman et al,20 there was no change in the ability to isolate HIV by culture after GM-CSF administration. In an extension of that trial, no consistent change in serum HIV p24 antigen was observed in patients receiving long-term GM-CSF administered by the subcutaneous route.45 Thus, there is not a clear consensus at this time on whether treatment of HIV-infected patients with GM-CSF (without concomitant AZT administration) results in an increase in their serum p24 antigen. Moreover, it is not known what the clinical significance would be of an increase in HIV p24 antigen, and such a potential effect must be balanced against the clinical benefits of GM-CSF in a given patient. It would at a minimum seem prudent to monitor HIV replication if GM-CSF is used in HIV-infected patients without concomitant antiretroviral therapy, and further clinical studies will be necessary to further investigate this issue. The data reported here cannot, in themselves, be used to argue for or against the use of GM-CSF or M-CSF by themselves in various clinical settings associated with AIDS.

In regard to the use of these cytokines with dideoxynucleosides, it is worth stressing that even in cultures stimulated by GM-CSF or M-CSF, complete inhibition of HIV replication could be attained with concentrations of AZT, ddC, or ddl that are attainable in patients. Recently, several trials exploring the simultaneous use of AZT and GM-CSF have been conducted. Preliminary results from these trials have provided evidence of an anti-HIV effect with this regimen, and it has been observed that certain patients who develop bone marrow toxicity when receiving AZT as a single agent can tolerate the combination of AZT and GM-CSF (and Pluda JM, Broder S, Yarchooan R, unpublished data, September 1990). Nevertheless, it is impossible to tell from these early, uncontrolled results whether an actual enhancement of activity of AZT has been observed in its use with GM-CSF. One problem in interpreting such feasibility studies of GM-CSF in HIV infection has been that because of the interest in using GM-CSF to reverse neutropenia, most of the patients studied to date have had advanced AIDS and extensive prior therapy with AZT. Such patients may intrinsically respond poorly to AZT therapy,18,19,46 and it is difficult to compare the results with other phase 1 trials enrolling different patient populations. Additional controlled studies will be needed to further assess the role of this combination in the treatment of AIDS.

In regard to the use of ddC or ddl with GM-CSF, or any dideoxynucleoside with M-CSF, it is again worth stressing that suppression of HIV replication could be attained in vitro at drug concentrations that are attainable in vivo, and these in vitro results should not deter clinical investigators from exploring the cytokines for their bone marrow stimulatory activities in this setting. A similar extrapolation can be drawn for Epo and G-CSF, neither of which appeared to have any effects on either HIV replication or the anti-HIV activities of the agents tested. Indeed, clinical trials of these latter two agents suggest that they can enhance erythroid and granulocyte bone marrow function, respectively, in AIDS patients without stimulating HIV replication.22,40 In summary, the results of these studies indicate that bone marrow stimulatory cytokines may have complex in vitro effects on the replication of HIV and the anti-HIV activity of various dideoxynucleosides in M/M that appear to result from a complex interplay of factors and that cannot be predicted a priori. These observations may have clinical implications inasmuch as the cytokines under discussion not only are being used as therapeutic agents in HIV infection but also are produced endogenously. However, there are numerous factors that may influence the replication of HIV in such patients. All of the cytokines described in this report have significant clinical potential in various diseases, including AIDS, and additional research will be needed to further delineate the precise clinical implications of the present observations.

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Effects of bone marrow stimulatory cytokines on human immunodeficiency virus replication and the antiviral activity of dideoxynucleosides in cultures of monocyte/macrophages

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