

# Synergistic Activity of Granulocyte-Macrophage Colony-Stimulating Factor and 3'-Azido-3'-Deoxythymidine against Human Immunodeficiency Virus In Vitro

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The ability of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 3'-azido-3'-deoxythymidine (AZT) to inhibit human immunodeficiency virus (HIV) in the U-937 monocytic cell line was examined. Acutely HIV-infected U-937 cells were exposed to GM-CSF (0.03, 0.3, 3.0, or 30.0 U/ml) and AZT (0.1, 1.0, or 10.0  $\mu$ M) alone and in combination for 14 to 17 days. Reverse transcriptase activity in the supernatant, the percentage of cells expressing viral antigens by indirect immunofluorescence, and the 50% tissue culture infectious dose per milliliter of supernatant were determined to assess the level of viral replication in treated and control cultures. By the fractional-product method of analysis, nearly all combinations of GM-CSF and AZT synergistically inhibited HIV replication by these three measurements. The most effective combinations were 30 U of GM-CSF per ml with 0.1, 1.0, or 10.0  $\mu$ M AZT. These treatments resulted in no reverse transcriptase activity in the supernatants, <1% immunofluorescent positive cells, and <8 50% tissue culture infectious doses per ml in the absence of cytotoxicity. Despite this degree of suppression, productive viral replication returned in all cultures within 4 to 10 days after drug removal. Combined therapy with GM-CSF and AZT merits consideration in the approach to HIV-associated illnesses.

A number of agents have been described that show activity against human immunodeficiency virus (HIV) in vitro. These include suramin, antimoniotungstate (HPA 23), ribavirin, phosphonoformate, alpha interferon, and 3'-azido-3'-deoxythymidine (AZT) among others (4, 10, 13-16, 22, 24). Of these agents, AZT has received the most interest because of its specificity as an inhibitor of the reverse transcriptase (RT) of HIV (5), its in vitro activity against the virus (16), and, most importantly, its clinical efficacy. In a preliminary clinical trial, AZT was shown to improve T-cell numbers and skin test reactivity in a group of patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) (25), and a recent double-blind, placebo-controlled trial among patients with AIDS or ARC was terminated early because of significantly diminished mortality in the treated group (*Med. Lett. Drugs Ther.*, 28:107-109, 1986). AZT was the first drug licensed for certain HIV-related disorders.

The encouraging results with AZT to date are only a first step in the control of HIV-related diseases, however. Efforts continue to discover more effective, less toxic compounds. One group of agents that is of ongoing interest are cytokines, as they are natural products of the immune system that may possess immunoenhancing as well as antiviral properties. We have recently reported that one such cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), has substantial HIV-inhibitory activity in the U-937 monocytic cell line (7). Even in the absence of any direct anti-HIV activity, this compound is of interest as potential therapy for AIDS and ARC because of its hematopoietic-stimulatory activity. GM-CSF has been demonstrated to substantially increase leukocyte counts in a primate infected with a simian type D retrovirus (3) and is now in clinical trials in humans

with HIV infection and bone marrow suppression (J. Groopman, personal communication).

As the number of agents with potential efficacy in treating HIV infections increases, the logical extension of the therapeutic approach is to consider the use of agents in combination, both to enhance efficacy and to diminish potential toxicity. Synergistic anti-HIV activity has recently been reported for alpha interferon in combination with phosphonoformate or AZT in lymphoid cell systems (8, 9). Given the increasing importance of the monocyte/macrophage system in the pathogenesis of HIV infection (6, 11, 12, 17, 21), we have attempted to extend our previous findings by examining the activity of GM-CSF in combination with AZT in the HIV-infected U-937 monocytic cell system.

## MATERIALS AND METHODS

**Virus strain and cell lines.** The HTLV-III<sub>B</sub> strain of HIV (courtesy R. Gallo, Bethesda, Md.) was propagated in H9 cells (19). H9 cells were maintained in RPMI 1640 medium supplemented with penicillin (250 U/ml), streptomycin (250  $\mu$ g/ml), 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and 20% heat-inactivated fetal calf serum (M.A. Bioproducts, Walkersville, Md.). The U-937 cell line (7) was maintained in RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine, and 10% fetal calf serum. Both cell lines were mycoplasma free. Cell viability was determined by the trypan blue dye exclusion technique.

**Compounds.** Recombinant human GM-CSF was obtained from Genetics Institute (Cambridge, Mass.). It had a specific activity of 10<sup>7</sup> U/mg of protein and >99% purity. One unit of GM-CSF is defined as the amount that produces a half-maximal response in a colony-forming assay in which an internal laboratory reference standard is included. This

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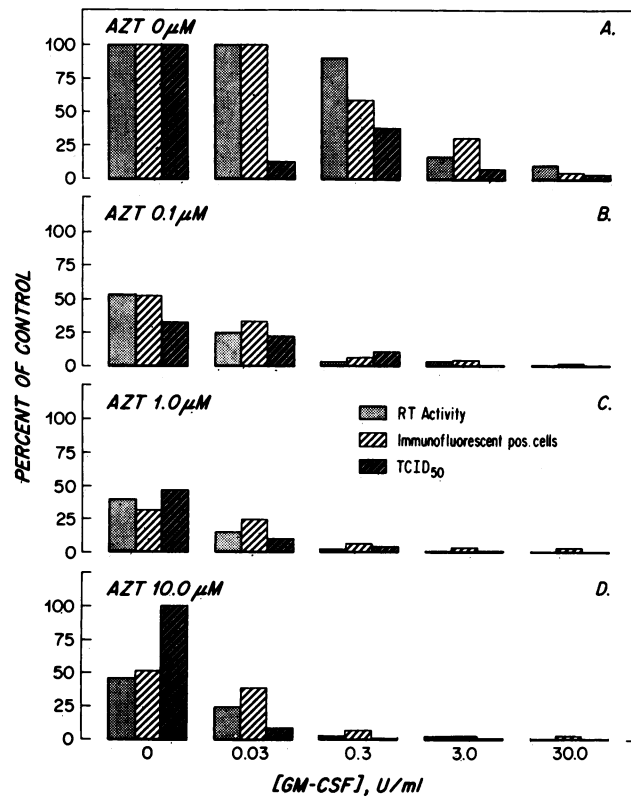


FIG. 1. Effect of AZT and GM-CSF on HIV replication in U-937 cell cultures. The fraction of the control value for RT activity, immunofluorescent positive cells (for HIV antigens), and supernatant infectious virus (TCID<sub>50</sub>) is represented for all combinations of AZT and GM-CSF at concentrations of 0, 0.1, 1.0, and 10.0 μM and 0.0, 0.03, 0.3, 3.0, and 30.0 U/ml, respectively.

activity was measured in the laboratories of Genetics Institute.

AZT was obtained from Burroughs Wellcome (Research Triangle Park, N.C.) in powdered form. It was dissolved in RPMI medium with 10% fetal calf serum and stored in aliquots at a concentration of 1 mM at -70°C.

**Infection and antiviral agent treatment.** Uninfected U-937 cells were suspended to a concentration of  $2 \times 10^5$ /ml and incubated with medium without GM-CSF or with GM-CSF at 0.03, 0.3, 3.0, or 30.0 U/ml for 4 days in 24-well plates (Costar, Cambridge, Mass.). They were then infected by incubating  $5 \times 10^6$  cells in 1 ml of filtered, cell-free supernatant from an HTLV-III<sub>B</sub>-infected H9 or U-937 cell culture for 2 h at 37°C. The mean RT activity of the inocula was  $10^6$  cpm/ml. Control cells were mock infected with a filtered, cell-free supernatant from an uninfected H9 or U-937 cell culture. Following the period of virus adsorption, the cell cultures were pelleted and suspended to a concentration of  $2 \times 10^5$  cells per ml in control medium or medium containing AZT at 0.1, 1.0, or 10.0 μM. GM-CSF was replenished in cultures which had been pretreated with this compound. Every 3 to 4 days the cultures were split and suspended to a concentration of  $2 \times 10^5$  cells per ml in fresh medium containing no drug, GM-CSF, AZT, or GM-CSF and AZT as originally treated. Cultures were maintained in this fashion for 14 to 17 days until the supernatant RT in untreated, HIV-infected cultures reached a level greater than  $10^6$  cpm/ml. At this point all cultures were sampled for supernatant RT activity, immunofluorescence for HIV antigens, and

supernatant virus yield as described below. The cultures were then pelleted and suspended in medium without GM-CSF or AZT and monitored for 14 days after drug removal. The results reported represent the mean values for two experiments repeated in their entirety with reproducible results.

**RT activity.** RT activity in the supernatant was determined by standard techniques following virion precipitation by polyethylene glycol (18).

**Immunofluorescence.** Expression of HIV antigens was determined by indirect immunofluorescence. At the times indicated, infected and control cells were washed in phosphate-buffered saline, air-dried on glass slides, and fixed in 50% methanol-50% acetone. Slides were incubated with a known-positive anti-HIV human serum for 30 min at 37°C in a humidified atmosphere. They were then stained with a fluorescein isothiocyanate-conjugated F(ab')<sub>2</sub> goat anti-human immunoglobulin (Cooper Biomedical, Malvern, Pa.). After an Evans blue counterstain, the slides were examined in a nonblinded fashion under an Olympus BH-2 fluorescence microscope, and the percentage of immunofluorescent positive cells was quantitated.

**TCID<sub>50</sub> determinations.** Assays of culture supernatants for virus yield were performed as follows. Cell-free supernatants from the cultures were initially diluted 1:8 and placed in the first column of a 96-well microtiter plate (Costar, Cambridge, Mass.), and serial 1:4 dilutions were made across the plate. H9 cells ( $4 \times 10^4$ ) were then added to each microtiter well to give a total volume of 200 μl/well. The plates were split 1:2 every 3 to 4 days, and after 10 to 11 days of incubation the endpoint was determined by the observation of cytopathic effects. Each sample was run in six replicates, and the 50% endpoint (i.e., 50% of the wells at a given dilution demonstrating cytopathic effects) was calculated by the method of Reed and Muench (20). The results are expressed as 50% tissue culture infectious dose (TCID<sub>50</sub>) per milliliter of original culture supernatant.

**Evaluation of combined antiviral effect.** The fractional-product method was used to determine whether synergistic antiviral effects were present in cultures treated with GM-CSF and AZT in combination (1, 23). Analyses were performed for supernatant RT activity, percentage of immunofluorescent positive cells, and virus yield on the day of drug removal. These three parameters were determined for the HIV-infected, untreated cultures and are termed virus control levels for RT, immunofluorescence, and TCID<sub>50</sub>, respectively. The fraction of these virus control values produced in cultures treated with GM-CSF or AZT alone was then calculated. The products of fractions calculated for exposure to either agent alone were then compared with the fraction of the virus control determined experimentally for that particular drug combination. If the observed (i.e., experimental) value for the agents in combination is less than the product of the fractions for the two agents when used alone, then the activity is synergistic.

## RESULTS

**Individual treatments.** HIV-infected, untreated U-937 cultures demonstrated mean values for supernatant RT activity, percentage of immunofluorescent positive cells, and supernatant infectious virus production of  $1.8 \times 10^6$  cpm/ml, 63%, and 11,425 TCID<sub>50</sub>/ml, respectively. The effect of exposure to GM-CSF alone on HIV infection of U-937 cells is illustrated in Fig. 1A. A dose-dependent decrease in viral replication was seen at the concentration range examined (0.03 to

TABLE 1. Effects of AZT and GM-CSF on HIV replication in acutely infected U-937 cell cultures

AZT ( $\mu\text{M}$ )	GM-CSF (U/ml)	RT activity			Immunofluorescent positive cells			Virus yield		
		cpm/ml	Calculated f.p. <sup>a</sup>	Actual fraction <sup>b</sup>	%	Calculated f.p.	Actual fraction	TCID <sub>50</sub> /ml	Calculated f.p.	Actual fraction
0.0	0.0	$1.8 \times 10^6$		1.00	63		1.00	11,425		1.00
	0.03	$2.1 \times 10^6$		1.20	73		1.16	1,348		0.12
	0.3	$1.6 \times 10^6$		0.91	37		0.59	4,389		0.38
	3.0	$3.0 \times 10^5$		0.17	20		0.31	860		0.08
	30.0	$1.9 \times 10^5$		0.11	3		0.05	164		0.014
0.1	0.0	$9.2 \times 10^5$		0.53	33		0.52	3,672		0.32
	0.03	$4.4 \times 10^5$	0.64	0.25 <sup>s</sup>	21	0.60	0.33 <sup>s</sup>	2,511	0.04	0.22
	0.3	$5.7 \times 10^4$	0.48	0.03 <sup>s</sup>	4	0.31	0.06 <sup>s</sup>	1,097	0.12	0.10 <sup>s</sup>
	3.0	$4.3 \times 10^3$	0.09	0.002 <sup>s</sup>	3	0.16	0.04 <sup>s</sup>	36	0.03	0.003 <sup>s</sup>
	30.0	— <sup>c</sup>	0.06	0.00 <sup>s</sup>	<1	0.03	<0.016 <sup>s</sup>	<8	0.004	<0.0007 <sup>s</sup>
1.0	0.0	$6.9 \times 10^5$		0.39	20		0.31	5,240		0.46
	0.03	$2.6 \times 10^5$	0.47	0.15 <sup>s</sup>	15	0.36	0.24 <sup>s</sup>	995	0.06	0.09
	0.3	$2.8 \times 10^4$	0.35	0.02 <sup>s</sup>	4	0.18	0.06 <sup>s</sup>	464	0.17	0.04 <sup>s</sup>
	3.0	$4.4 \times 10^3$	0.07	0.003 <sup>s</sup>	2	0.10	0.03 <sup>s</sup>	16	0.04	0.001 <sup>s</sup>
	30.0	—	0.04	0.00 <sup>s</sup>	<1	0.02	<0.016 <sup>s</sup>	<8	0.006	<0.0007 <sup>s</sup>
10.0	0.0	$7.9 \times 10^5$		0.45	32		0.51	15,716		1.38
	0.03	$4.2 \times 10^5$	0.54	0.24 <sup>s</sup>	24	0.59	0.38 <sup>s</sup>	879	0.17	0.08 <sup>s</sup>
	0.3	$4.3 \times 10^4$	0.41	0.02 <sup>s</sup>	4	0.30	0.06 <sup>s</sup>	51	0.53	0.004 <sup>s</sup>
	3.0	$9.3 \times 10^3$	0.08	0.005 <sup>s</sup>	1	0.16	0.02 <sup>s</sup>	49	0.11	0.004 <sup>s</sup>
	30.0	—	0.05	0.00 <sup>s</sup>	<1	0.03	<0.016 <sup>s</sup>	<8	0.02	<0.0007 <sup>s</sup>

<sup>a</sup> The calculated fractional product (f.p.) is the product of the fraction of the control value (RT activity, immunofluorescent positive cells, or TCID<sub>50</sub>) achieved when AZT or GM-CSF was used alone at the indicated concentrations.

<sup>b</sup> Fraction of the control value achieved experimentally when AZT and GM-CSF were used together at the indicated concentrations. Superscript s indicates synergy, as determined by the fractional-product method (see text).

<sup>c</sup> —, Not detected.

30 U/ml). Maximal effects were seen at 30 U/ml, with reductions of 89, 95, and 98.6% in RT, immunofluorescence, and TCID<sub>50</sub>, respectively.

The effects of AZT alone are illustrated in Fig. 1B, C, and D (first set of bars). Maximal effects on RT activity and immunofluorescence were seen at 1  $\mu\text{M}$ , with reductions of 61 and 69%, respectively. The maximal reduction in supernatant infectious virus yield (TCID<sub>50</sub>) was 68%, seen at a concentration of 0.1  $\mu\text{M}$ . Paradoxically, AZT alone at 10  $\mu\text{M}$  was consistently less effective than at 1  $\mu\text{M}$  by all parameters examined.

**Combination treatments.** (i) **RT activity.** The effect of combining AZT at 0.1, 1.0, and 10.0  $\mu\text{M}$  with GM-CSF at 0.03, 0.3, 3.0, and 30.0 U/ml is shown in Fig. 1 and Table 1. By the fractional-product determination, all combinations of these agents examined resulted in synergistic reductions in RT activity (Table 1). Increasing efficacy was seen with increasing concentrations of both agents. Reductions of 97 to 98%, >99% and 100% were seen when AZT (0.1 to 10  $\mu\text{M}$ ) was used with GM-CSF at 0.3, 3, and 30 U/ml, respectively.

(ii) **Immunofluorescence for HIV antigens.** The results of combination GM-CSF-AZT treatment on viral antigen expression was similar to that seen on RT activity (Fig. 1 and Table 1). All combinations resulted in synergistic reductions in the percentage of immunofluorescent positive cells (Table 1). AZT (0.1 to 10  $\mu\text{M}$ ) addition to 0.3, 3.0, and 30.0 U of GM-CSF per ml resulted in reductions of 94, 96-98, and >98%, respectively.

(iii) **TCID<sub>50</sub>.** The effect of combination antiviral treatment on infectious virus yield is also represented in Fig. 1 and Table 1. Except for the lowest concentration of GM-CSF (0.03 U/ml) with 0.1 or 1.0  $\mu\text{M}$  AZT, synergistic reductions were evident with all combinations examined (Table 1). Greater than 99% reductions were found with AZT (0.1 to 10

$\mu\text{M}$ ) combined with 3 U of GM-CSF per ml, and >99.9% reductions were noted with AZT in combination with 30 U of GM-CSF per ml.

**Cell viability.** Total viable cell counts and percent viable cells for all cultures examined on the day of drug removal are listed in Table 2. These values were lowest for the HIV-infected, untreated culture,  $4.4 \times 10^5$  cells per ml and 53%, respectively. All treated cultures showed improved cell survival and no evidence of compound-induced cytotoxicity.

**Compound removal.** Following removal of the agents from the treated cultures, there were gradual rises in the supernatant RT activity and the percentage of immunofluorescent positive cells. The return to control levels was, in general, inversely proportional to the degree of suppression of viral replication noted on the day of compound removal; that is, the cultures that demonstrated the greatest degree of inhibition demonstrated the greatest delay in return to productive viral infection (Fig. 2). The cultures that were maximally suppressed, with no supernatant RT activity, <1% immunofluorescent positive cells, and <8 TCID<sub>50</sub>/ml, became productive again 4 to 10 days after drug removal. Thus, neither cure nor prevention of HIV infection was achieved in this cell line.

## DISCUSSION

The effort currently under way to control HIV infection is proceeding along three avenues of investigation aimed at developing specific inhibitors of viral replication, biologic response modifiers which will strengthen the host immune response, and an effective vaccine (24). Potentially complicating the search for effective antiviral therapy are recent advances in our understanding of the pathogenesis of HIV

TABLE 2. Effect of AZT and GM-CSF on cell viability in HIV-infected and uninfected U-937 cell cultures

HIV infected	AZT ( $\mu$ M)	GM-CSF (U/ml)	Viable cell count ( $\log_{10}$ /ml)	% Viable cells
+	0.0	0.0	5.64	53
		0.03	6.06	67
		0.3	6.09	90
		3.0	6.17	95
		30.0	6.15	92
+	0.1	0.0	6.17	91
		0.03	6.08	94
		0.3	6.16	96
		3.0	6.12	95
		30.0	6.12	95
+	1.0	0.0	6.17	95
		0.03	6.13	96
		0.3	6.10	96
		3.0	6.19	95
		30.0	6.08	91
+	10.0	0.0	5.98	92
		0.03	6.18	94
		0.3	6.18	96
		3.0	6.10	94
		30.0	6.28	91
-	0	0	6.28	98
	10.0	30.0	6.14	96

infection—specifically, the important role of the monocyte/macrophage as a primary target of infection and a potential reservoir for the ongoing infection of other cell types (6, 11, 12, 17, 21). The important role of cells of this lineage led us to use the monocytic cell line U-937 in antiviral studies. This line has phenotypic and functional similarities to monocytes and has been shown by a number of investigators to be susceptible to HIV infection, most probably because it is CD4 antigen positive (2, 12). We recently reported that GM-CSF has inhibitory activity on HIV replication in both persistently and acutely infected U-937 cells (7). Cells that were pretreated with 30 to 300 U/ml and continuously exposed postinfection demonstrated marked

inhibition of supernatant RT activity and the percentage of cells expressing HIV antigens. In the present studies we have attempted to extend these studies by examining (i) the effects of very low concentrations of GM-CSF, (ii) the efficacy of AZT in a monocyte model of HIV infection, and (iii) the nature of the combined effect of these two agents on HIV replication.

Our results demonstrate a dose-response effect of GM-CSF on HIV replication at low concentrations and the efficacy of AZT in a nonlymphoid cell system. The inhibitory effect of AZT alone in this system was modest, however, and was less effective at 10  $\mu$ M than 1.0  $\mu$ M. This observation was consistent among all three parameters of virus replication that were examined. Two additional experiments have confirmed that the inhibitory effect of AZT alone in the HIV/U-937 system reaches a plateau or diminishes at concentrations of 1 to 10  $\mu$ M when RT activity, immunofluorescent positive cells, or HIV p24 antigen level in culture supernatants are examined. These results differ from those reported for lymphoid cell systems (16) and is unexplained. It is possibly related to limitation of AZT phosphorylation in U-937 cells.

Most importantly, the combination of these two agents at nearly all concentrations examined was synergistic when analyzed by the fractional-product method for supernatant RT activity, expression of viral antigens by fixed-cell immunofluorescence, and infectious virus yield. The most effective combinations in these studies were GM-CSF at 30 U/ml with 0.1, 1, or 10  $\mu$ M AZT. These combinations resulted in no supernatant RT activity, <1% antigen-positive cells, and <8 TCID<sub>50</sub>/ml (100, >98.4, and >99.9% reductions, respectively). Despite the near absence of viral expression in the cultures most effectively suppressed, return of virus replication within 4 to 10 days of drug removal occurred in all cases. A similar phenomenon was described previously for GM-CSF used alone in concentrations as high as 300 U/ml (7). Thus, GM-CSF and AZT, either alone or in combination, successfully suppress but do not prevent initial HIV infection or its reemergence in this cell system.

The mechanism of inhibition of HIV replication by AZT has been well described (5), but that of GM-CSF is unclear. Preliminary data suggest that it is not mediated by interferon production or oxidative metabolism (R. Rose, personal communication). Whether it is mediated by a unique anti-

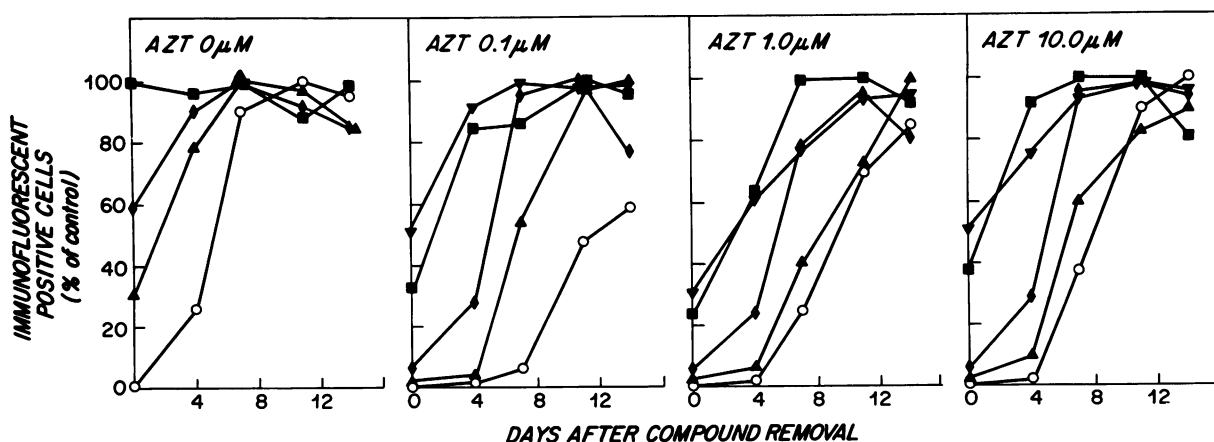


FIG. 2. Immunofluorescent positive cells for HIV antigens in U-937 cell cultures following removal of AZT and GM-CSF. The percentage of value obtained in control cultures (HIV-infected but untreated) is represented for each treated culture followed for 14 days after compound removal. Panels represent cultures treated with AZT at 0.0, 0.1, 1.0, or 10.0  $\mu$ M as indicated. Cultures also treated with GM-CSF at 0.0 ( $\blacktriangledown$ ), 0.03 ( $\blacksquare$ ), 0.3 ( $\blacklozenge$ ), 3.0 ( $\blacktriangle$ ), or 30.0 ( $\circ$ ) U/ml.

ral mechanism or through effects on cellular differentiation is the subject of active investigation.

Both AZT and GM-CSF have been or are being examined in human trials among individuals with HIV infection. The initial rationale for the use of GM-CSF is its potential utility as a multilineage hematopoietin in HIV-infected persons who demonstrate marrow suppression as a manifestation of their infection (3). However, it may also be useful in preventing or correcting the marrow toxicity seen in individuals treated with AZT (25; *Med. Lett. Drugs Ther.*, 1986), and if it possesses *in vivo* anti-HIV activity, GM-CSF might well enhance the clinical benefits seen thus far in early AZT trials. The data presented here have demonstrated synergistic antiviral effects of these two compounds *in vitro* at concentrations which are achievable in humans and support the consideration of clinical trials of GM-CSF in combination with AZT in persons with AIDS or ARC.

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