

Inhibition of immune functions by antiviral drugs.

W Heagy, ... , P A Lopez, R W Finberg

J Clin Invest. 1991;**87**(6):1916-1924. <https://doi.org/10.1172/JCI115217>.

Research Article

Immune functions were evaluated in vitro for PBMC isolated from healthy donors and cultured with the antiviral agents, 3'-azido-3'-deoxythymidine (AZT), ribavirin, ganciclovir, 2'3'-dideoxyinosine (ddI), or acyclovir. To identify methods for assessing the effects of antiviral drugs on immune cells, the PBMC response to mitogens, Con A, or phytohemagglutinin was evaluated from measurements of [3H]thymidine and [14C]-leucine incorporation, cell growth, cellular RNA, DNA, and protein levels, and the PBMC proliferative cycle (i.e., progression from G0----G1----S----G2 + M). At clinically relevant concentrations, AZT, ribavirin, or ganciclovir diminished PBMC responsiveness to mitogen. The numbers of proliferating cells in G1, S, and G2 + M phases of the cell cycle, DNA content, and [3H]thymidine uptake were decreased in cultures treated with AZT, ribavirin, or ganciclovir. AZT or ribavirin but not ganciclovir reduced RNA and protein in the cultures and inhibited cell growth. Whereas AZT, ribavirin, or ganciclovir were antiproliferative, ddI or acyclovir had little, if any, effect on PBMC mitogenesis. The inhibitory effects of antivirals on immune cells may contribute to the immune deterioration observed in patients following prolonged use of the drugs.

Find the latest version:

<https://jci.me/115217/pdf>



Inhibition of Immune Functions by Antiviral Drugs

Wyrta Heagy,^{*†} Clyde Crumpacker,[‡] Peter A. Lopez,^{*†} and Robert W. Finberg^{*†}

^{*}Laboratory of Infectious Diseases, Dana-Farber Cancer Institute, and [†]Departments of Medicine and [‡]Pathology, Harvard Medical School, Boston, Massachusetts 02115; [§]Division of Infectious Disease, Harvard Thorndike-Dana Research Laboratory and Departments of Medicine, Harvard Medical School and Beth Israel Hospital, Boston, Massachusetts 02215; and [¶]Laboratory of Flow Cytometry, Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Abstract

Immune functions were evaluated in vitro for PBMC isolated from healthy donors and cultured with the antiviral agents, 3'-azido-3'-deoxythymidine (AZT), ribavirin, ganciclovir, 2'-dideoxyinosine (ddI), or acyclovir. To identify methods for assessing the effects of antiviral drugs on immune cells, the PBMC response to mitogens, Con A, or phytohemagglutinin was evaluated from measurements of [³H]thymidine and [¹⁴C]leucine incorporation, cell growth, cellular RNA, DNA, and protein levels, and the PBMC proliferative cycle (i.e., progression from G0 → G1 → S → G2 + M).

At clinically relevant concentrations, AZT, ribavirin, or ganciclovir diminished PBMC responsiveness to mitogen. The numbers of proliferating cells in G1, S, and G2 + M phases of the cell cycle, DNA content, and [³H]thymidine uptake were decreased in cultures treated with AZT, ribavirin, or ganciclovir. AZT or ribavirin but not ganciclovir reduced RNA and protein in the cultures and inhibited cell growth. Whereas AZT, ribavirin, or ganciclovir were antiproliferative, ddI or acyclovir had little, if any, effect on PBMC mitogenesis. The inhibitory effects of antivirals on immune cells may contribute to the immune deterioration observed in patients following prolonged use of the drugs. (*J. Clin. Invest.* 1991; 87:1916-1924.)
Key words: lymphocytes • virus replication • HIV • immunosuppression • herpes virus

Introduction

At present there are few safe and effective drugs for treatment of viral diseases. Although antiviral activity has been demonstrated in vitro for the nucleoside analogues 3'-azido-3'-deoxythymidine (Zidovudine; Zidovir) (AZT),¹ ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazole), ganciclovir (9-[1,3-dihydroxy-2-propoxymethyl] guanine; DHPG; BIOLF-62; 2'NDG), and 2'-dideoxyinosine (ddI), neither their full therapeutic potential nor range of toxicity has been evaluated (1-6). By virtue of their resemblance to thymidine, adenosine, and guanosine, such nucleoside analogues are likely to have adverse effects on mammalian DNA, RNA, and/or

protein synthesis, in addition to their effects on viral replication (2). Previous studies have shown many synthetic nucleosides to be toxic on in vivo use (2); however, acyclovir (9-[2-hydroxyethoxymethyl]guanine) which has low in vivo and in vitro toxicity, is a notable exception (1). Acyclovir is now widely used in renal and bone marrow transplant recipients for prophylaxis and treatment of herpes simplex virus and varicella zoster virus infections (1).

To date, AZT is the only approved drug for treatment of HIV disease. Recent studies with ddI, however, have documented clinical improvements in HIV infected patients and even low dose therapy with ddI has resulted in statistically significant increases in CD4 cells and decreases in HIV p24 antigenemia (7-9). Ribavirin has been used in treatment of respiratory syncytial virus infection (10), influenza (11), and Lassa fever (12), and ribavirin, like AZT and ddI, is currently under evaluation for its effectiveness in treatment of HIV disease (13, 14). Ganciclovir, which has activity against many herpes group viruses, is the first effective drug for treatment of cytomegalovirus retinitis in patients with AIDS (15).

The effects of nucleoside antiviral drugs on immune function have not been well defined. Although established cell lines have been cultured with the antivirals (16), the findings from such studies may not always reflect the activity of the drugs on freshly isolated immune response cells. Some investigators have analyzed PBMC responses in cultures treated with the nucleoside antivirals, but these studies have relied on uptake of labelled DNA/RNA precursors to assess the effects exerted by the drugs (4, 16-18). Since these antivirals probably alter intracellular nucleotide pools (2), the levels of incorporation for DNA/RNA precursors may not parallel the degree of DNA/RNA synthesis in the cells, and consequently, may not be valid indicators of cellular functions. For these reasons we have used a series of assays to evaluate, in vitro, the effects of AZT, ribavirin, ganciclovir, acyclovir, and ddI on the immune response of PBMC obtained from normal, healthy donors. PBMC responsiveness to T cell mitogens, Con A, or PHA, was measured and the effects of the antiviral drugs were assessed on the PBMC uptake of [³H]thymidine and [¹⁴C]leucine, blastogenesis, the RNA, DNA, and protein content of activated cultures and stages of the proliferative cycle for lectin-stimulated lymphocytes. Our studies show that AZT, ribavirin, or ganciclovir were inhibitory for cultured PBMC, diminishing their responsiveness to mitogens.

Methods

Isolation of PBMC. PBMC were separated from whole, heparinized blood obtained by venipuncture from healthy volunteer donors. 30-ml portions of blood were layered over 15 ml of lymphocyte separation medium (Organon Teknica Corp., Durham, NC) and after centrifugation, cells at the interface were collected, and then washed twice in

Address reprint requests to Wyrta Heagy, Ph.D., Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

Received for publication 22 October 1990 and in revised form 28 January 1991.

1. Abbreviations used in this paper: ARC, AIDS related complex; AZT, 3'-azido-3'-deoxythymidine; ddI, 2'-dideoxyinosine; FI, fluorescence; HO, Hoechst 33342; PY, pyronin Y; R640, rhodamine 640.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/91/06/1916/09 \$2.00

Volume 87, June 1991, 1916-1924

HL-1 culture medium (Ventrex Laboratories, Inc., Portland, ME) supplemented with 2 mM glutamine, and 100 U/ml penicillin-streptomycin (Gibco Laboratories, Grand Island NY).

Antiviral drugs. Sources for the antivirals used in these studies were: AZT (Burroughs Wellcome Co., Research Triangle Park, NC); ribavirin (Viratek, Inc., Costa Mesa, CA); ganciclovir (Syntex Laboratories Inc., Palo Alto, CA); acyclovir (Burroughs Wellcome Co.); and ddI (Calbiochem-Behring Corp., La Jolla, CA). These agents were prepared in HL-1 medium as stock solutions at $2-4 \times 10^{-3}$ M and serially diluted before assay.

[3 H]thymidine uptake. PBMC (10^5) were dispensed in 100- μ l portions of culture medium to 96-well flat-bottom microdilution plates (Falcon Labware, Becton, Dickinson, & Co., Oxnard, CA) and then activated by the addition of 50 μ l of an 8- μ g/ml preparation of Con A (Sigma Chemical Co., St Louis, MO) or PHA (Difco Laboratories Inc., Detroit, MI). The antiviral drugs were added in 50- μ l vols and the cultures then incubated for 3 d at 37°C in a 5% CO₂ atmosphere. Subsequently, [3 H]thymidine (50 μ l; 1 μ Ci) (New England Nuclear, Boston, MA) was added, and the incubations continued for an additional 12 h. In studies designed to measure the kinetics for [3 H]thymidine incorporation the cells were incubated for periods of 2–6 d; [3 H]thymidine was present for the final 4 h of the culture period. Incubations were terminated by aspiration of culture medium and transfer of the PBMC to filters by use of a cell harvester. Subsequently, the filters were dried and then counted in a liquid scintillation counter.

Two-color analysis of the cell cycle. Cultures were initiated by the transfer of 10^6 PBMC suspended in 1 ml of HL-1 medium to 24-well plates (Costar, Data Packaging Corp., Cambridge, MA). PBMC were activated by the addition of 2 μ g/ml (final) of Con A, the antiviral drugs were added, and then the cultures were incubated at 37°C in a 5% CO₂ atmosphere for 3 d. Subsequently, PBMC were labelled for DNA and RNA by use of the double-staining method previously described by Shapiro (19). Cells (10^6 /ml) were suspended in a 5- μ M solution of the DNA-specific dye Hoechst 33342 (HO) (Sigma Chemical Co.), dissolved in HL-1 medium, and then incubated for 45 min at 37°C in a 5% CO₂ atmosphere. The RNA-specific fluorochrome pyronin Y (PY) (Aldrich Chemical Co., Milwaukee, WI) was then added (5 μ M, final concentration) and the incubations continued for an additional 45 min. Following two washes with cold PBS supplemented with 5 μ M PY, cells were fixed with 2% formalin and stored in the dark at 4°C until analyzed.

DNA and RNA content of the PBMC was analyzed by dual fluorescence flow cytometry as previously described (19, 20). HO blue fluorescence (FI) and PY red FI were simultaneously recorded from individual cells using a 455 BP and 610 filter, respectively. Data were collected and stored in list mode using a dual laser Epics 750 Series flow cytometer (Coulter Electronics Inc., Hialeah, FL). Subsequently, the data were transferred to an HP 9000 computer for analysis and display using LYSYS software (Becton Dickinson Immunocytometry Systems, Mountain View, CA) with cell cycle positions determined according to methods described by Darzynkiewicz and Traganos (20, 21).

Blast cells formed in response to lectin. PBMC were activated with Con A and cultured with or without the antiviral drugs for 3 d as described above (see: *Two-color analysis of the cell cycle*). Large lymphocytes were distinguished from small resting ones by the analysis of forward and 90° light scatter parameters (22) acquired on an Epics 750 series flow cytometer using MDADS based software (Coulter Electronics, Inc.).

Protein assays. Protein content for the PBMC was measured by the technique of Crissman and Steinkamp (23). PBMC (10^6 /ml) were suspended in a solution of 5 μ g/ml (final) rhodamine 640 perchlorate (R640) (Exciton, Inc., Dayton, Ohio) dissolved in culture medium and then incubated for 90 min at 37°C in a 5% CO₂ incubator. Subsequently, the cells were fixed with 2% formalin and stored in the dark at 4°C until analyzed. Protein content for the R640 stained cells was determined by flow cytometric analysis. R640 emission was recorded using a 570 long pass filter on an Epics 750 series flow cytometer and analyzed using MDADS based software (Coulter Electronics, Inc.).

Protein synthesis was measured from incorporation of [14 C]leucine (New England Nuclear) into the cellular components precipitated by 5% cold TCA. PBMC (10^6) were harvested from cultures after a 3-d incubation with Con A (2 μ g/ml) or the lectin + antiviral and transferred in 1-ml portions of the culture supernatant to 5-ml test tubes. 20 μ l (1 μ Ci) of [14 C]leucine was added to each tube and the tubes were incubated for 5 h at 37°C in a 5% CO₂ incubator. Cells were then pelleted by centrifugation, culture medium removed by aspiration, and the cell pellets washed three times in 2 ml of cold 5% TCA. Subsequently, the precipitate was dissolved in 1 ml of a solution containing 10^{-3} M EDTA, 10^{-3} M NaHCO₃, and 2% SDS, transferred to 10 ml of Biofluor liquid scintillant (New England Nuclear), and then counted in a scintillation counter.

Results

AZT, ribavirin, or ganciclovir, but not acyclovir or ddI, were potent inhibitors of [3 H]thymidine uptake in cultures of lectin-activated PBMC. The addition of AZT, ribavirin, or ganciclovir to cultures of Con A-activated PBMC resulted in a marked decrease in uptake of [3 H]thymidine (Fig. 1, A–C). At low doses (i.e., ≤ 4 μ M) AZT, ribavirin, or ganciclovir diminished [3 H]thymidine incorporation and high doses (40–400 μ M) abrogated the uptake of this labelled DNA precursor (Fig. 1, A–C). Whereas low doses of AZT, ribavirin, or ganciclovir reduced labelling, acyclovir caused only a modest decrease (i.e., on average a 50% reduction) in [3 H]thymidine uptake at the 400- μ M dose (Fig. 1, A–D). Interestingly, ddI, unlike the other antivirals, had little, if any, effect on the [3 H]thymidine incorporated in PBMC cultures (Fig. 1, A–E).

[3 H]thymidine incorporation for PBMC stimulated with PHA, like that for Con A, was markedly reduced for cultures incubated with AZT, ribavirin, or ganciclovir (data shown for AZT and ddI; see Fig. 1 F). Acyclovir caused a modest impairment in labelling at the 400- μ M dose and ddI had no effect on [3 H]thymidine uptake in PHA or Con A-activated cultures (Fig. 1 E and F).

The kinetics for [3 H]thymidine uptake by the PBMC were unchanged in cultures incubated with the antivirals. The data of Fig. 1 F show the effects of AZT and ddI on the time course for [3 H]thymidine uptake in PBMC cultures established from a single donor and activated with PHA (1 μ g/ml); labelling peaked on day 4 and waned by day 6 in cultures incubated with or without drugs and, therefore, the effects of the antivirals on [3 H]thymidine uptake were not merely the result of a shift in the kinetics of the lectin response.

AZT, ribavirin, or ganciclovir, but not acyclovir or ddI, were potent inhibitors of the PBMC proliferative response to lectin. Upon stimulation with mitogen lymphocytes enter the proliferative cycle; quiescent cells (G0) undergo change to an activated state (G1), then progress through subsequent stages (S, G2) to mitosis (M), and in turn, the resulting daughter cells enter the cycle (24). To measure lymphocyte proliferation and to assess the effects exerted by antiviral drugs on this immune function we determined the numbers of resting G0 or cycling cells in the G1, S, G2, or M phases of the proliferative cycle in cultures of Con A-stimulated PBMC. Cell position (G0, G1, S, G2 + M) in the cycle was ascertained from measurements of DNA and RNA. A double labelling technique was used to differentially stain DNA with one fluorochrome and RNA with another, and then, the DNA and RNA content for individual cells was measured by dual fluorescence flow cytometry. DNA was labelled with the DNA-specific HO dye that stains viable cells and

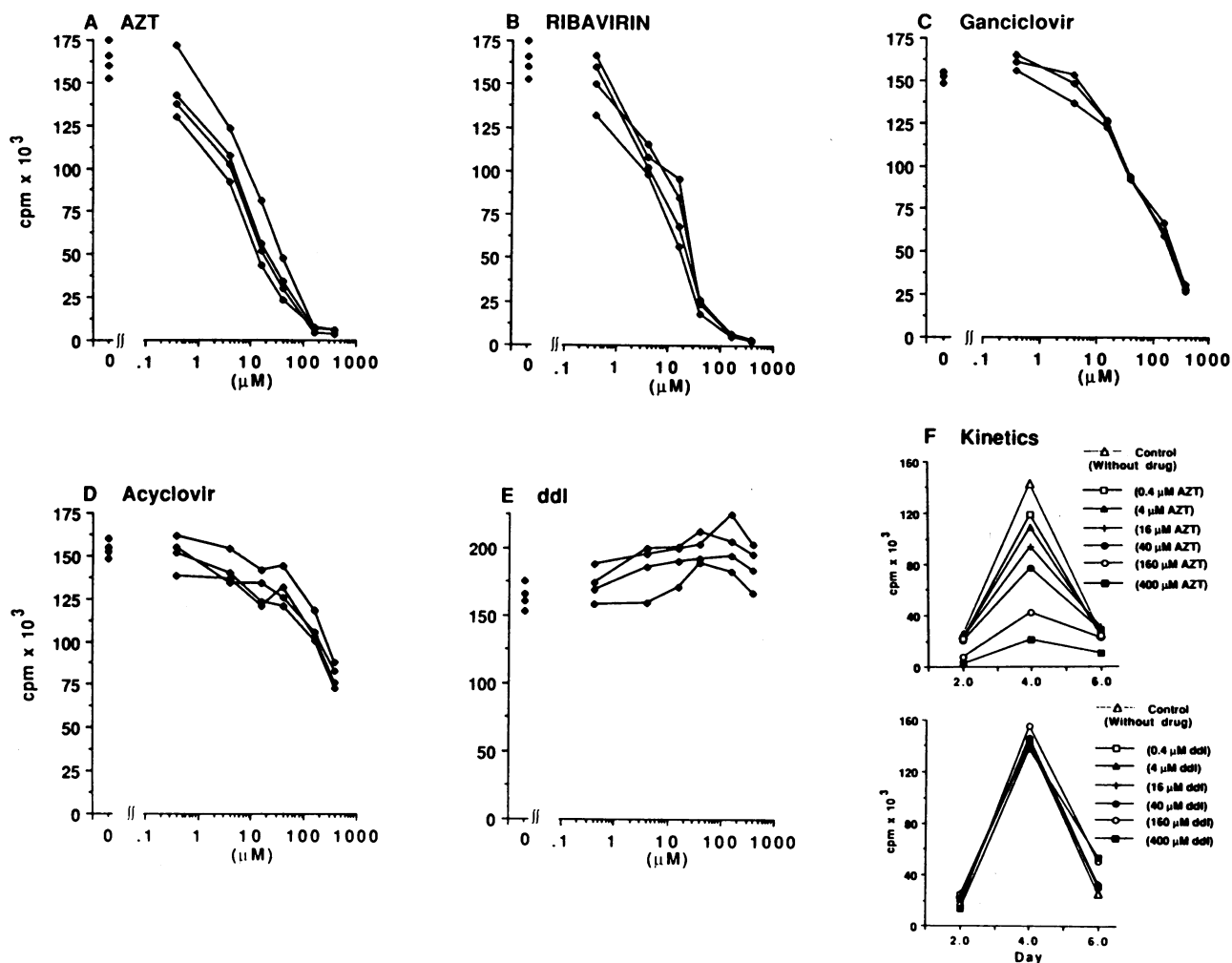


Figure 1. AZT, ribavirin, or ganciclovir, but not acyclovir or ddI, were potent inhibitors of $[^3\text{H}]$ thymidine uptake in lectin-stimulated cultures of PBMC. PBMC (10^5) were activated by the addition of $2 \mu\text{g}/\text{ml}$ of Con A (A-E) or PHA (F) and cultured with the antiviral drugs at the concentrations indicated for 3 d (A-E) or (as shown for panel F) periods of 2, 4, or 6 d. $[^3\text{H}]$ thymidine ($1 \mu\text{Ci}$) was present in the cultures for the final 12 h (A-E) or 4 h (F) of the culture period. All data points were determined from the arithmetic means of results obtained for four replicate cultures. (Standard deviations for the mean values were $< 5\%$.)

under the conditions employed for these studies (described in Methods) is a stoichiometric stain for DNA (19). PY was employed for staining cytoplasmic RNA. Activated PBMC that were synthesizing DNA (S) or that had completed a round of synthesis, but as yet not separated into daughter cells (G2 + M), were distinguished by virtue of their greater DNA content from nonactivated, quiescent cells (G0). Cells in G1, defined as those synthesizing RNA but not DNA, were readily distinguished from those in G0 (resting), S, or G2 + M since DNA and RNA were simultaneously measured for individual cells. This double staining technique and dual fluorescence flow cytometry enabled us to enumerate the cells in G0, G1, S, or G2 + M compartments of the cycle and thereby to measure proliferation in the cultures. By then comparing the proliferative response (i.e., number of cells in the compartments of the cycle) for drug-treated and nontreated cultures, we determined the antiviral effects on PBMC proliferation.

Cultures established from five donors are shown as two parameter histograms in Fig. 2, which depicts the position of cells in the proliferative cycle as defined by their relative DNA and

RNA content. Data of Fig. 2 are presented for control cultures in which the PBMC were maintained in medium alone, for Con A-stimulated cultures incubated without drugs, and for Con A-stimulated cells that were treated with AZT, ribavirin, ganciclovir, acyclovir, or ddI. Data for the Con A cultures incubated with or without drugs are also included in the line graphs of Fig. 3. As expected, nonstimulated PBMC incubated in medium alone (see histograms labelled Medium, nonstimulated; Fig. 2) were almost exclusively positioned in G0 (Fig. 2). Since Con A-stimulated cultures (see histograms labelled Con A-drug, Fig. 2) had entered the proliferative cycle, cells were observed in the G1, S, G2 + M, as well as G0 compartment, a distribution pattern characteristic for proliferating lymphocytes (24).

Addition of AZT, ribavirin, or ganciclovir to the Con A-activated cultures resulted in aberrations in the proliferative cycle (Figs. 2 and 3). As can be clearly seen in the histograms depicting the effects of AZT (Fig. 2) at doses as low as $0.4 \mu\text{M}$ AZT interrupted the progression of cells through the proliferative cycle (i.e., from G0 to G1, S to G2 + M and G2 + M to G0). At

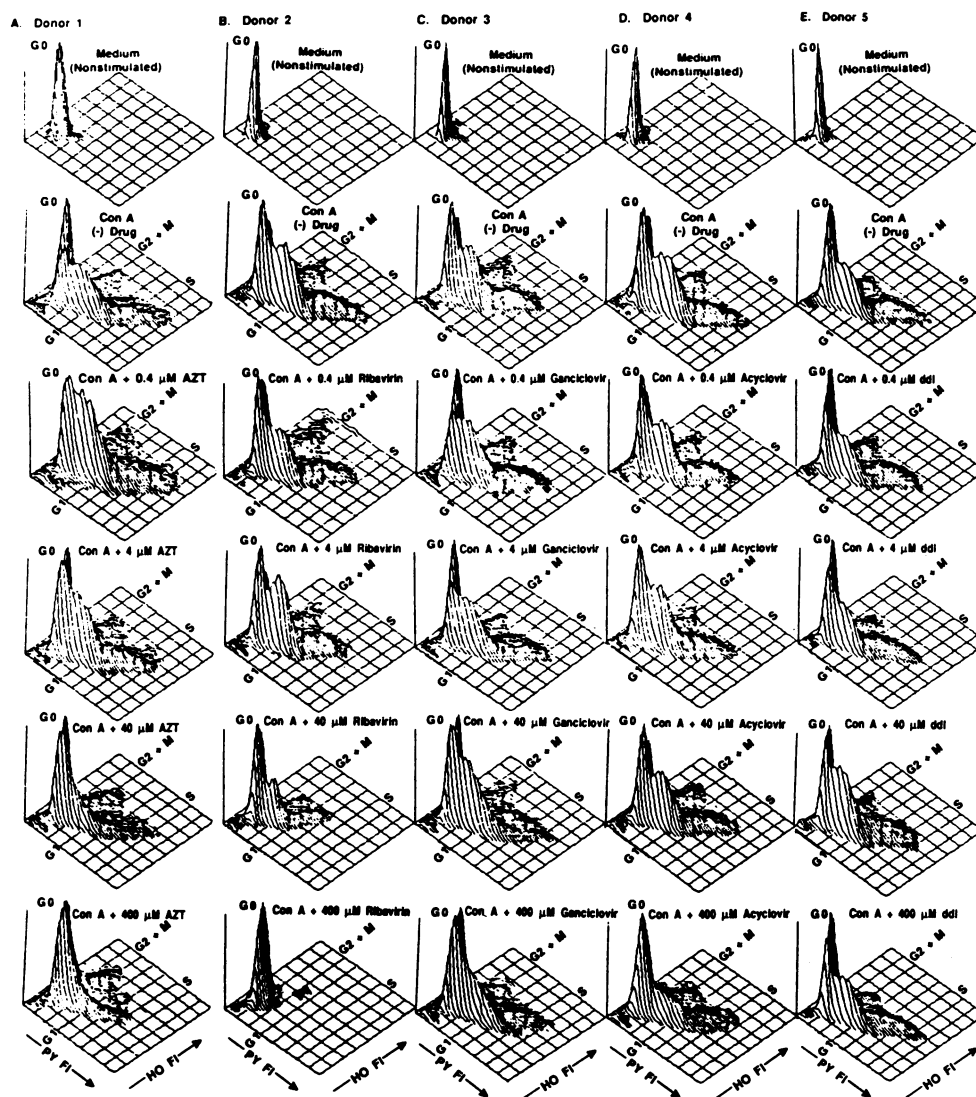


Figure 2. AZT, ribavirin, or ganciclovir, but not acyclovir or ddI, caused marked abnormalities in the proliferative cycle of Con A-activated PBMC. PBMC (10^6) were cultured in medium alone, Con A ($2 \mu\text{g/ml}$), or lectin + antiviral drug for 3 d. Cells were then double labelled with fluorochromes specific for DNA (HO) and RNA (PY). HO blue FI and PY red FI were measured by dual fluorescence flow cytometry. Data are presented as two parameter histograms representing the distribution of PBMC in relation to their relative DNA (HO FI) and RNA (PY FI) content. Histograms depict distributions for 25,000 cells.

$0.4 \mu\text{M}$ AZT the boundary between G0 and G1 phases was obliterated and, in addition, the S and G2 + M compartments were enlarged with an excessive number of cells. At increased doses ($\geq 4 \mu\text{M}$) AZT diminished the DNA content of the cultures and caused deficiencies in the S and G2 + M compartments of the proliferative cycle (Figs. 2 and 3).

Inspection of the histograms presented in Fig. 2 shows that ribavirin, like AZT, induced anomalies in the PBMC proliferative cycle. Ribavirin at $0.4 \mu\text{M}$ caused an enlargement in the G2 + M compartment suggesting a defect in either the transition from G2 to M or in cell division (M) itself (Figs. 2 and 3). When the ribavirin concentration was increased 10-fold to $4 \mu\text{M}$ there were anomalies in G1 and S, and when further increased 100-fold to $40 \mu\text{M}$, the total numbers of cycling cells measured for G1, S, and G2 + M were markedly reduced (Figs. 2 and 3).

Ganciclovir at doses of $4 \mu\text{M}$ caused a detectable, albeit modest, decrease in the S and G2 + M compartments of the proliferative cycle (Fig. 2). When the ganciclovir concentration in the cultures was increased by 10-fold to $40 \mu\text{M}$ the numbers of cells in the S and G2 + M phases of the cycle were further

reduced. At $400 \mu\text{M}$ ganciclovir the numbers of cycling cells in G1, S, and G2 + M compartments diminished (Figs. 2 and 3).

Whereas low doses (0.4 to $4 \mu\text{M}$) of AZT, ribavirin, or ganciclovir caused abnormalities in the proliferative cycle (Figs. 2 and 3), equivalent concentrations of acyclovir or ddI had little, if any, effect on the proliferative response of PBMC (Figs. 2 and 3). In fact, acyclovir or ddI were only inhibitory at high doses ($\geq 40 \mu\text{M}$) (Figs. 2 and 3).

Our studies in which the proliferative cycle was analyzed by two-color staining for DNA and RNA clearly showed that incubation of lectin-stimulated PBMC with AZT, ribavirin, or ganciclovir resulted in abnormalities in the cell cycle and diminished proliferation. These findings are in agreement with our data in which PBMC proliferation was measured indirectly by [^3H]thymidine incorporation (Fig. 1) and therefore they serve to substantiate our results obtained with the labelled DNA precursor.

AZT or ribavirin, but not ganciclovir, acyclovir, or ddI, were potent inhibitors of PBMC transformation to Con A blasts. Lectin-stimulated transformation of lymphocytes from small (resting) to large blast cells was markedly diminished in PBMC

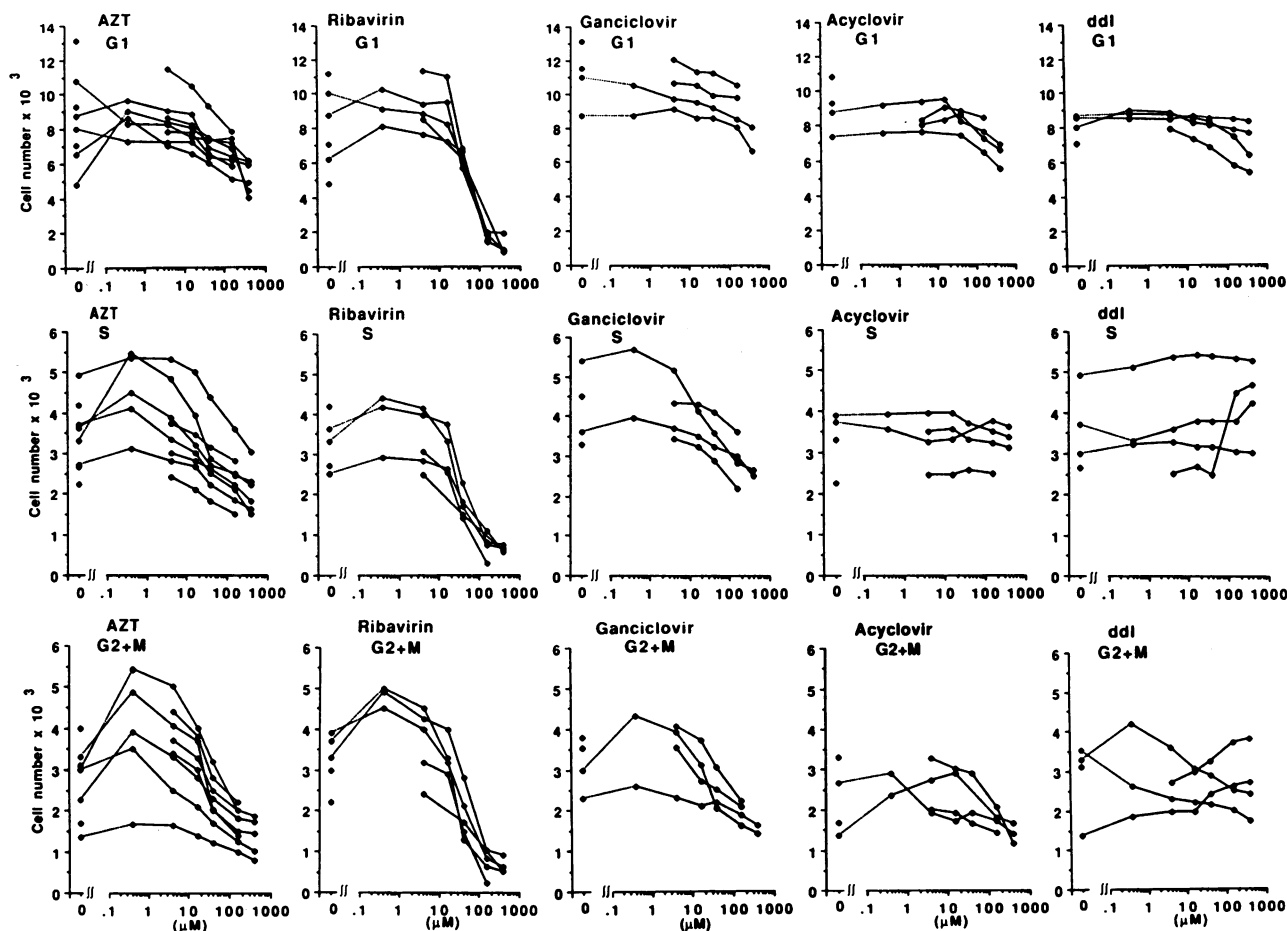


Figure 3. PBMC progression through the cell cycle was markedly impaired by AZT, ribavirin, or ganciclovir, but not acyclovir or ddI. DNA and RNA content for PBMC incubated with Con A or Con A + antiviral drug was measured by double staining and dual fluorescence flow cytometry as described for Fig. 2. Cell cycle position (G1, S, and G2 + M) was then determined for the PBMC from measurements of their DNA and RNA as described for Fig. 2; 25,000 cells were analyzed for each culture. Data are shown as number of cells in the sample in G1 (upper panels), S (middle panels), and G2 + M (lower panels) compartments of the proliferative cycle vs. drug concentration. The broken lines connect values measured for PBMC incubated with a 0.4- μ M dose of drug to the matched controls cultured without drug (i.e., 0 concentration); these lines point out the effects of 0.4- μ M doses of the antivirals on the PBMC obtained from individual donors.

cultures incubated with AZT or ribavirin, but not ganciclovir, acyclovir, or ddI (Fig. 4). AZT, at doses as low as 4 μ M, decreased the number of Con A blasts in PBMC cultures, and as the concentration of AZT was increased, blast formation was further diminished (Fig. 4 A). Ribavirin, like AZT, inhibited blastogenesis; inhibition was detected in cultures incubated with 12 μ M ribavirin, and the response was abolished in cultures incubated with 400 μ M ribavirin (Fig. 4 B).

It is noteworthy that we employed cytofluorometry (i.e., measured forward and right angle light scatter) to measure the number of blast cells in the cultures, and therefore, these measurements were obtained without aid of DNA precursors (e.g., [3 H]thymidine) or other labelling agents (e.g., HO stain for DNA). These studies eliminate the possibility that the effects measured for AZT or ribavirin on PBMC responsiveness to lectin resulted solely from artifacts caused by way of drug competition with nucleoside precursors or other interference with labelling procedures.

Whereas AZT and ribavirin were inhibitory, ganciclovir, acyclovir, or ddI (data shown for ganciclovir or ddI; Fig. 4, C

and D) had only a modest, if any, effect on blastogenesis. Only at high doses (400 μ M) did ganciclovir, acyclovir, or ddI cause any diminution in the number of blast cells in the Con A-stimulated cultures (Fig. 4, C and D).

Ribavirin or AZT, but not ganciclovir, acyclovir, or ddI, reduced the protein content of Con A-activated PBMC. Although ribavirin was the more potent inhibitor, the incubation of Con A-activated PBMC with ribavirin or AZT diminished the protein content of the cultures (Fig. 5, A and B). The drug concentration that resulted in a 50% reduction in protein (I_{50}) was 100 μ M for ribavirin and 400 μ M for AZT; the inhibitory effect titrated to 4- μ M doses of ribavirin or AZT (Fig. 5, A and B). Neither acyclovir nor ddI, on the other hand, reduced the level of protein in the lectin-activated cultures at doses < 400 μ M (Fig. 5, A and B).

The decreased levels of protein in the ribavirin-treated cultures resulted from a decline in protein synthesis since incorporation of [14 C]leucine into the protein fraction precipitated by TCA was reduced in cultures incubated with ribavirin (Fig. 6). [14 C]leucine uptake was diminished at doses of 4 μ M ribavirin

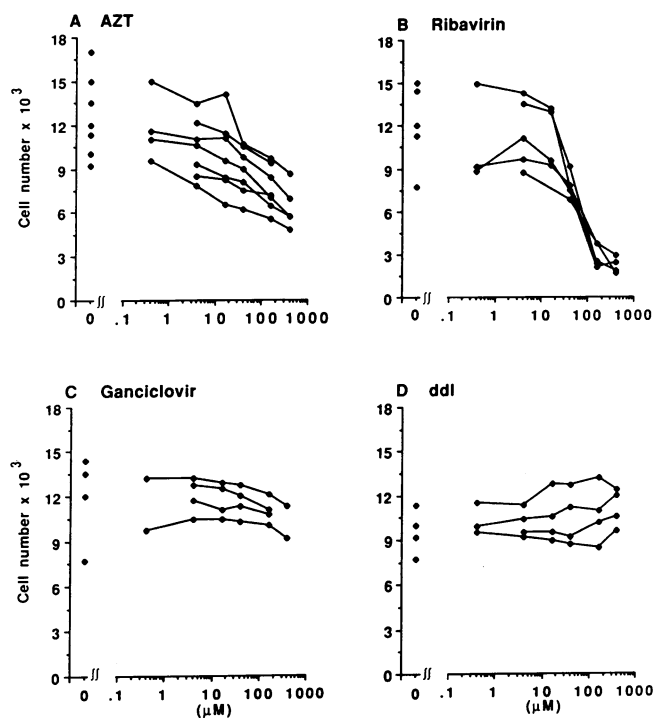


Figure 4. Con A-stimulated blastogenesis was inhibited in PBMC cultures incubated with AZT or ribavirin, but not ganciclovir, acyclovir, or ddI. Con A cultures were initiated and then incubated for 3 d as described for Fig. 2. Con A blasts were distinguished from small resting cells in the cultures by flow cytometry and analysis of forward and 90° degree light scatter. Samples of 25,000 cells from each culture were analyzed; data are shown as numbers of blast cells in the samples.

and was abrogated when the ribavirin dose was increased to 400 μM (Fig. 6). Unlike ribavirin, neither ganciclovir nor acyclovir diminished protein synthesis since neither agent reduced the level of [¹⁴C]leucine in TCA precipitates (Fig. 6).

Discussion

Recovery from viral disease is closely linked to immune function, especially T lymphocyte activity (25–27), and since synthetic nucleoside antivirals have potential toxicity, particularly on rapidly dividing cells, we have studied the effects of AZT, ribavirin, ganciclovir, ddI, and acyclovir on mitogen-stimulated immune cells. The responsiveness of PBMC isolated from normal, healthy donors was measured for cultures incubated with or without these agents.

As expected, our findings showed that acyclovir, an antiviral drug that has been extensively studied in vitro and in vivo (1, 17), had little effect on PBMC responsiveness to mitogen (Figs. 1–3, 5). Toxicity was observed only with high acyclovir doses; intravenous administration of acyclovir doses of 5–15 mg/kg body wt result in plasma levels of 40–92 μM (28) and our studies showed that at these concentrations the PBMC proliferative response was reduced only 10–20% (Figs. 1–3, 5). Interestingly, ddI had minimal toxicity even in cultures treated with high (supratherapeutic) doses of drug (Figs. 1–5).

Whereas the PBMC response to Con A was largely unaffected by acyclovir or ddI, AZT, ribavirin, or ganciclovir diminished the lectin response (Figs. 1–6). The addition of AZT,

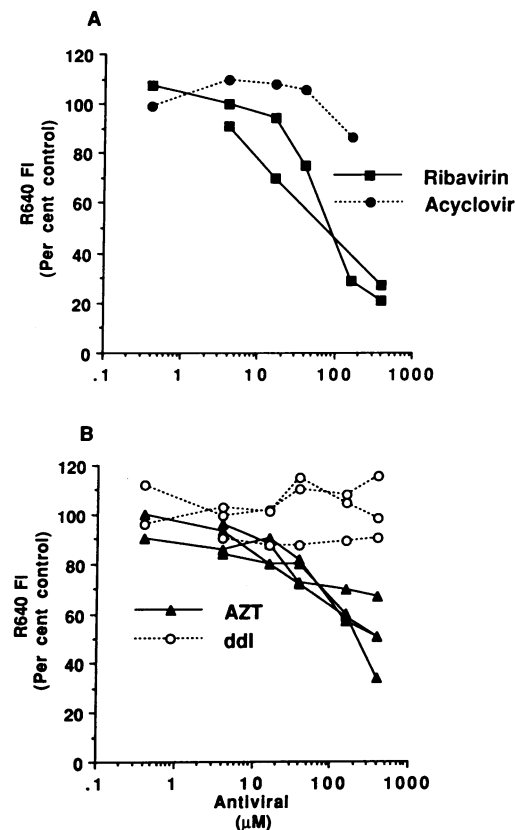


Figure 5. Protein content for Con A-activated PBMC was diminished by AZT or ribavirin, but not ganciclovir, acyclovir, or ddI. PBMC were activated with Con A and cultured with or without the drugs as described for Fig. 2. Cell protein was labelled by incubation of the PBMC with the fluorogenic dye R640. Red FI emitted from cell associated R640 was measured by flow cytofluorometry and the number of Con A-activated cells that had undergone protein synthesis was determined from comparisons of the R640 FI emitted from stimulated and nonstimulated (PBMC maintained in culture medium alone) cultures. The effects of the antivirals on the protein content of activated cultures was measured by comparisons between PBMC incubated with or without drugs. Samples of 25,000 cells from each culture were analyzed. Data are shown as per cent control for R640 FI [(number of cells with increased R640 FI in Con A + drug cultures)/(number of cells with increased red FI in Con A – drug cultures) × 100%].

ribavirin, or ganciclovir to the PBMC cultures resulted in the decreased uptake of [³H]thymidine (Fig. 1) and quantitative defects in the proliferative cycle (Figs. 2 and 3). It is noteworthy that exposure to low doses (0.4–4 μM) of AZT, ribavirin, or ganciclovir impaired lymphocyte functions since in vitro cultured cells are exposed to a fixed drug concentration; however, in vivo the circulating level of drug may be rapidly diminished.

AZT or ribavirin, but not ganciclovir caused marked decreases in the growth of Con A blasts (Fig. 4), and the RNA (Figs. 2 and 3) and protein content (Fig. 5) of activated cells. Bowden et al. (18) reported previously that addition of ganciclovir to PBMC cultures reduced [³H]thymidine incorporation but not elaboration of lymphokines, an immune function that is dependent upon RNA and protein synthesis. Sommadossi and Carlisle (29) reported that the in vitro addition of ganciclovir to human hematopoietic progenitor cells, including granu-

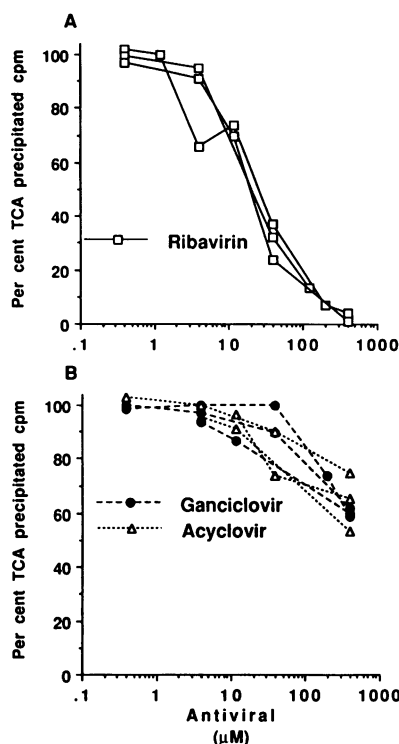


Figure 6. Ribavirin inhibited uptake of [^{14}C]-leucine in Con A-stimulated cultures of PBMC. PBMC (10^6) were activated with 2 $\mu\text{g}/\text{ml}$ of Con A and incubated for 3 d with the antiviral drugs at the concentrations indicated. Data are shown as per cent [^{14}C]leucine incorporated into TCA precipitated cpm: [(cpm for Con A + drug cultures)/(cpm for Con A - drug) \times 100%]. All data points were determined from the arithmetic means for four replicate cultures. Values measured for the Con A cultures incubated without any drug were between 15.5 and 18.0×10^3 cpm and spontaneous uptake of [^{14}C]leucine in the

PBMC cultures maintained in medium alone was $\leq 2.5 \times 10^3$ cpm. Data were obtained in three separate experiments with cells from three individual donors.

locytes, macrophages, and erythrocytes decreased their growth. Taken together these studies indicate that ganciclovir may be toxic for rapidly dividing populations of blood cells and their precursors. Our findings show that the effects of ganciclovir are targeted primarily on the S and subsequent G2 + M (but not G1) phases of the proliferative cycle (Figs. 2 and 3), and therefore, the ganciclovir toxicity probably results from its effect(s) on DNA synthesis.

Our studies show that AZT is a potent inhibitor of the PBMC response to mitogens; this drug decreased cell uptake of [^3H]thymidine, caused defects in the cell cycle, reduced growth of Con A blasts, and diminished the protein content in the cultures (Figs. 1–6). Mitsuya et al. (16) studied the effects of AZT on the growth of an established T helper cell line (ATH8) and they reported little difference between the viability for drug treated or nontreated cultures. It is possible that the established T cell line used for their studies was resistant to AZT toxicity since such resistance has been reported for other established cell lines (22, 30). Mitsuya et al. (16) also presented data on [^3H]uridine uptake into RNA for mitogen-stimulated PBMC established from one healthy donor. Inspection of these data showed that at 10 μM AZT caused a detectable reduction in the level of [^3H]uridine incorporated in PHA or Con A-activated cultures. Our studies, in which the RNA content of PBMC was determined by fluorescent staining and flow cytometry, showed that AZT diminished RNA; this effect was dose dependent and the inhibition titrated to AZT doses of 4 μM (see Fig. 3).

At present AZT is the only drug demonstrated to be effective in treatment of HIV infection; however, long term or high

dose therapy with AZT has resulted in severe toxicity, particularly anemia and neutropenia (31). Peak plasma levels attained for high dose AZT range between 6 and 10 μM (32). We find that AZT caused abnormalities in the proliferative cycle of lectin-stimulated PBMC at concentrations as low as 0.4 μM (Figs. 2 and 3), and our studies, as well as those of Mitsuya et al. (16), have indicated that therapeutic levels of AZT inhibit RNA synthesis in PBMC cultures. Taken together, these findings suggest that AZT may adversely affect immune functions dependent on proliferation or RNA and protein synthesis (e.g., elaboration of lymphokines). In AIDS, where viral replication occurs in immune response cells, it is possible that the toxicity resulting from such an antiviral may be enhanced. Since patients with HIV disease have cellular immune deficiencies at the outset of treatment, any agent that further depresses the immune response may, particularly over the long term, provide little value to the patient.

Recent studies (33, 34) have shown that in patients with AIDS or the AIDS related complex (ARC) low doses (300 mg) of AZT cause less toxicity than higher doses (600–1,500 mg) but provide the same clinical and virologic effects as the higher doses. Fischl et al. (33) reported that in the AIDS patients treated with AZT the numbers of CD4 cells are transiently increased but subsequently diminished after 8 wk of therapy. The studies of Collier et al. (34) have shown that in patients with ARC the greatest improvement in mean CD4 lymphocyte counts was in patients treated with low doses of AZT. It is noteworthy that our studies point out the dose-dependent effects of AZT on lymphocytes, and therefore our findings would have predicted less toxicity with the low dose of AZT as has been recently demonstrated in clinical trials (33, 34). The methods used in our studies may be applicable to the study of other new antiviral compounds, and “prescreening” tests such as those we have used here could be performed before the clinical use of systemic antiviral agents.

We find that ribavirin, like AZT, diminished PBMC responsiveness to lectin. Our studies, as well as those of others (35), have shown that ribavirin inhibits [^3H]thymidine incorporation by cultured cells. Drach et al. (35) reported previously that in ribavirin-treated cultures [^3H]thymidine was not a reliable measure for DNA synthesis. These investigators found that in cultures of the KB cell line ribavirin inhibited [^3H]thymidine uptake by blocking the conversion of [^3H]-labelled deoxythymidine diphosphate to the [^3H]-labelled triphosphate and that this drug at concentrations $< 50 \mu\text{M}$ had little effect on cellular uptake of [^{32}P]phosphate. Although no data were presented, Drach et al. (35) reported that results obtained for Con A-stimulated lymphocytes were identical to those obtained for KB cells. Our studies show that ribavirin decreased protein synthesis (Fig. 6), diminished the capacity of lectin-stimulated PBMC to progress through the cell cycle (Figs. 2 and 3), and reduced blastogenesis (Fig. 4). Defects in the cell cycle resulted when PBMC were incubated with ribavirin doses of 0.4 μM ; at this low dose (0.4 μM) ribavirin caused activated PBMC to accumulate in the G2 + M compartment indicating a block at these stages of the proliferative cycle. At the higher 4- μM dose ribavirin reduced the number of cells in S, and as ribavirin was further increased to 40 μM , the numbers in G1 and G2 + M, as well as those in S, were diminished (Figs. 2 and 3) indicating that ribavirin was a potent inhibitor of the proliferative response. In addition to measuring [^3H]thymidine uptake, we

have quantitated the DNA, RNA, and protein content of cells, analyzed stages of the cell cycle, counted blasts, and measured protein synthesis in the cultures and ribavirin, by all methods employed, was inhibitory (Figs. 1–6). We have employed highly sensitive methods to assess the effects of ribavirin on the PBMC response to lectin and differences between the methodologies used by Drach et al. (35) and us most likely account for the disparities between these studies.

In clinical trials where patients with AIDS or ARC have been treated with oral ribavirin doses of 600–800 mg/d and in which steady-state serum levels were maintained between 8 and 10 μ M the p24 antigen was decreased and the CD4 cell number and lymphocyte proliferative response to lectin were enhanced (13, 36). With higher dose regimens of 1,200 to 1,600 mg/d of ribavirin the steady-state serum levels were greater than 13 μ M and the CD4 cell number, lymphoproliferation, or p24 antigen status showed no improvement (36). In fact, Roberts et al. (37) have reported that the lymphocyte counts decreased in the patient group treated with 1,200 to 1,600 mg/d of ribavirin suggesting possible lymphocyte toxicity. Interestingly, our studies show that ribavirin inhibition of the PBMC response to lectin was dose dependent and that responsiveness declined precipitously in cultures incubated with ribavirin concentrations > 12 μ M. It is possible that the treatment failure observed with higher ribavirin doses is linked to the toxicity of this agent on immune functions and that lower doses of the drug may avoid this toxicity.

It is noteworthy that our studies show AZT but not ddI to be a potent inhibitor of PBMC mitogenesis. Similar findings have been reported for bone marrow cell cultures (38); whereas low concentrations of AZT (i.e., 1.0 μ M) have inhibited myelopoiesis or erythropoiesis, ddI has been shown to have little effect on the growth of bone marrow progenitor cells (38–40). Mitsuya and Broder (4) reported that similar concentrations of ddI had no effect on the viability of an established T helper cell line. The major adverse effects noted in patients treated with ddI have been dose-dependent peripheral neuropathy, and in some patients, pancreatitis (9, 41). Early results from clinical studies show that p24 antigenemia has decreased and numbers of CD4 lymphocytes have increased in patients receiving ddI (9, 41). Possibly, ddI may prove safe and beneficial as an antiretroviral drug by avoiding lymphocyte toxicity. Further clinical study will determine the efficacy for ddI in HIV disease. Further in vitro studies of antiviral agents before their clinical use should allow us to make predictions about the consequences of these agents upon the host immune system.

Acknowledgments

Funding for this work was provided by grants from the National Institutes of Health (CA-34979, AI-29173, and AI-27659), United States Army Medical Research Development Contract (DAMD17-87-C-7151), Massachusetts Mutual Life Insurance Company, and a Biomedical Research Support Grant (S07 RR05526).

References

1. Dorsky, D. I., and C. S. Crumpacker. 1987. Drugs five years later: acyclovir. *Ann. Intern. Med.* 107:859–874.
2. Wood, M. J., and A. M. Geddes. 1987. Infection today: antiviral therapy. *Lancet*. ii:1189–1192.
3. Browne, M. J. 1978. Mechanism and specificity of action of ribavirin. *Antimicrob. Agents Chemother.* 15:747–753.
4. Mitsuya, H., and S. Broder. 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA*. 83:1911–1915.
5. Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA*. 83:8333–8337.
6. Ahluwalia, G., D. A. Conney, H. Mitsuya, A. Fridland, K. P. Flora, Z. Hao, M. Dalal, S. Broder, and D. G. Johns. 1987. Initial studies on the cellular pharmacology of 2',3'-dideoxyinosine, an inhibitor of HIV infectivity. *Biochem. Pharmacol.* 36:3797–3800.
7. Fauci, A. S. 1990. ddI—a good start, but still phase I. *N. Engl. J. Med.* 322:1386–1388.
8. Cooley, T. P., L. M. Kunches, C. A. Saunders, J. K. Ritter, C. J. Perkins, C. McLaren, R. P. McCaffrey, and H. A. Lieberman. 1990. Once-daily administration of 2',3'-dideoxyinosine (ddI) in patients with the acquired immunodeficiency syndrome or AIDS-related complex. *N. Engl. J. Med.* 322:1340–1345.
9. Yarchoan, R., H. Mitsuya, R. V. Thomas, J. M. Pluda, N. R. Hartman, C. F. Perno, K. S. Marczuk, J. P. Allain, D. G. Johns, and S. Broder. 1989. In vivo activity against HIV and favorable toxicity profile of 2',3'-dideoxyinosine. *Science (Wash. DC)*. 245:412–415.
10. Edelson, P. J. 1990. Clinical studies with ribavirin. *Pediatr. Infect. Dis. J.* 9(Suppl.):S71–73.
11. Knight, V., H. W. McClung, S. Z. Wilson, B. K. Waters, J. M. Quarles, R. W. Cameron, S. E. Greggs, J. M. Zerwas, and R. B. Couch. 1981. Ribavirin small-particle aerosol treatment of influenza. *Lancet*. ii:945–949.
12. McCormick, J. B., I. J. King, P. A. Webb, C. L. Scribner, R. B. Craven, K. M. Johnson, L. H. Elliott, and R. Belmont-Williams. 1986. Lassa fever: effective treatment with ribavirin. *N. Engl. J. Med.* 314:20–26.
13. Crumpacker, C. S., W. Heagy, G. Buley, J. E. Monroe, R. Finberg, S. Hussey, L. Schnipper, D. Lucey, T. H. Lee, M. F. McLane, et al. 1987. Ribavirin treatment of AIDS and ARC-A phase I study shows transient clinical improvement associated with suppression of HIV and enhanced lymphocyte proliferation. *Ann. Intern. Med.* 107:664–674.
14. Roberts, R. B., F. B. Hollinger, W. P. Parks, S. Rasheed, J. Laurence, P. N. R. Heseltine, R. W. Makuch, J. A. Lubina, K. M. Johnson, and the Ribavirin-LAS Collaborative Group. 1990. A multicenter clinical trial of oral ribavirin in HIV-infected people with lymphadenopathy: virologic observations. *AIDS (Lond.)*. 4:67–72.
15. Henderly, D. E., W. R. Freeman, D. M. Causey, and N. A. Rao. 1987. Cytomegalovirus retinitis and response to therapy with Ganciclovir. *Ophthalmology*. 94:425–434.
16. Mitsuya, H., R. F. Jarrett, M. Matsukura, F. Di Marzo Veronese, A. L. DeVico, M. G. Sarngadharan, D. G. Johns, M. S. Reitz, and S. Broder. 1987. Long-term inhibition of human T-lymphotropic virus type III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2',3'-dideoxynucleosides in vitro. *Proc. Natl. Acad. Sci. USA*. 84:2033–2037.
17. Levin, M. J., P. L. Leary, and R. D. Arbeit. 1980. Effect of acyclovir on the proliferation of human fibroblasts and peripheral blood mononuclear cells. *Antimicrob. Agents Chemother.* 17:947–953.
18. Bowden, R. A., J. Digel, E. C. Reed, and J. D. Meyers. 1987. Immunosuppressive effects of ganciclovir on in vitro lymphocyte responses. *J. Infect. Dis.* 156:899–903.
19. Shapiro, H. M. 1981. Flow cytometric estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and Pyronin Y. *Cytometry*. 2:143–150.
20. Darzynkiewicz, Z., and F. Traganos. 1990. Multiparameter flow cytometry in studies of the cell cycle. In *Flow Cytometry and Sorting*, 2nd ed. M. R. Melamed, T. Lindmo, and M. L. Mendelsohn, editors. Wiley-Liss, Div. of Wiley Publishing Group, New York. 469–501.
21. Traganos, F. 1990. Single- and multiparameter analysis of the effects of chemotherapeutic agents on cell proliferation in vitro. In *Flow Cytometry and Sorting*, 2nd ed. M. R. Melamed, T. Lindmo, and M. L. Mendelsohn, editors. Wiley-Liss, Div. of Wiley Publishing Group, New York. 773–801.
22. Bhalla, K. M., Birkhofer, L. Gongrong, S. Grant, W. MacLaughlin, J. Cole, G. Graham, and D. J. Volsky. 1989. 2'-Deoxycytidine protects normal human bone marrow progenitor cells in vitro against the cytotoxicity of 3'-azido-3'-deoxythymidine with preservation of antiretroviral activity. *Blood*. 74:1923–1928.
23. Crissman, H. A., and J. A. Steinkamp. 1981. Rapid, one step staining procedures for analysis of cellular DNA and protein by single and dual laser flow cytometry. *Cytometry*. 3:84–90.
24. Darzynkiewicz, Z., D. Evenson, L. Staiano-Coico, T. Sharpless, and M. R.

- Melamed. 1979. Relationship between RNA content and progression of lymphocytes through S phase of cell cycle. *Proc. Natl. Acad. Sci. USA*. 76:358-362.
25. Finberg, R. W., and R. Hom. 1986. The role of T cell immunity in infection with the herpes group viruses. In *The Year in Immunology*. J. M. Cruse and R. E. Lewis, Jr., editors. S. Karger AG, Basel. 267-278.
26. Ljungman, P., B. Lönnqvist, G. Gahrton, O. Ringdén, and B. Wahren. 1986. Cytomegalovirus-specific lymphocyte proliferation and in vitro cytomegalovirus IgG synthesis for diagnosis of cytomegalovirus infections after bone marrow transplantation. *Blood*. 68:108-112.
27. Buimovica-Klein, E., M. Lange, W. G. Ramey, M. H. Grieco, and L. Z. Cooper. 1987. Cell-mediated immune response in AIDS. *N. Engl. J. Med.* 311:328-329.
28. Whitley, R. J., M. R. Blum, N. Barton, and P. De Miranda. 1982. Pharmacokinetics of acyclovir in humans following intravenous administration: a model for the development of parental antivirals. Acyclovir symposium. *Am. J. Med.* 165-171.
29. Sommadossi, J. P., and R. Carlisle. 1987. Toxicity of 3'-azido-3'-deoxythymidine and 9-(1,3-dihydroxy-2-propoxymethyl) guanine for normal human hematopoietic progenitor cells in vitro. *Antimicrob. Agents Chemother.* 31:452-454.
30. Balzarini, J., R. Pauwels, M. Baba, P. Herdewijn, E. DeClercq, S. Broder, and D. J. Johns. 1988. The in vitro and in vivo anti-retrovirus activity and intracellular metabolism of 3'-azido-2',3'-deoxythymidine and 2',3'-dideoxycytidine are highly dependent on the cell species. *Biochem. Pharmacol.* 37:897-903.
31. Richman, D. D., M. A. Fischl, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, M. S. Hirsch, et al. 1987. The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N. Engl. J. Med.* 317:192-197.
32. Yarchoan, R., K. J. Weinhold, H. K. Lyerly, E. Gelmann, R. M. Blum, G. M. Shearer, H. Mitsuya, J. M. Collins, C. E. Myers, R. W. Klecker, et al. 1986. Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex. *Lancet*. i:575-580.
33. Fischl, M. A., C. B. Parker, C. Pettinelli, M. Wulfsohn, M. S. Hirsch, A. C. Collier, D. Antoniskis, M. Ho, D. D. Richman, E. Fuchs, et al. 1990. A randomized controlled trial of a reduced daily dose of zidovudine in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 323:1009-1014.
34. Collier, A. C., S. Bozzette, R. W. Coombs, D. M. Causey, D. A. Schoenfeld, S. A. Spector, C. B. Pettinelli, G. Davies, D. D. Richman, J. M. Leedom, et al. 1990. A pilot study of low-dose zidovudine in human immunodeficiency virus infection. *N. Engl. J. Med.* 323:1015-1021.
35. Drach, J. C., M. A. Thomas, J. W. Barnett, S. H. Smith, and C. Shipman, Jr. 1980. Tritiated thymidine incorporation does not measure DNA synthesis in ribavirin-treated human cells. *Science (Wash. DC)*. 212:549-551.
36. Crumpacker, C. S., G. Pearlstein, C. van der Horst, F. Valentine, S. Spector, and J. Mills. 1990. A phase one increasing dose trial ribavirin in patients with AIDS and ARC (ACTG 034). *Proc. VI International Conf. on AIDS, San Francisco* (Abstr.).
37. Roberts, R. B., K. Jurica, W. A. Meyer III, H. Paxton, and R. W. Makuch. 1990. A phase I study of ribavirin in human immunodeficiency virus-infected patients. *J. Infect. Dis.* 162:638-642.
38. Molina, J. M., and J. E. Groopman. 1989. Bone marrow toxicity of dideoxyinosine. *N. Engl. J. Med.* 321:1478.
39. Dainiak, N., M. Worthington, M. A. Riordan, S. Kreczko, and L. Goldman. 1988. 3'-Azido-3'-deoxythymidine (AZT) inhibits proliferation in vitro of human hematopoietic progenitor cells. *Br. J. Haematol.* 69:299-304.
40. Johnson, M., T. Caiazzo, J. M. Molina, R. Donahue, and J. Groopman. 1988. Inhibition of bone marrow myelopoiesis and erythropoiesis in vitro by anti-retroviral nucleoside derivatives. *Br. J. Haematol.* 70:137-141.
41. Lambert, J. S., M. Seidlin, R. C. Reichman, C. S. Plank, M. Lavery, G. D. Morese, C. Knupp, C. McLaren, C. Pettinelli, F. T. Valentine, and R. Dolin. 1990. 2',3'-Dideoxyinosine (ddI) in patients with the acquired immunodeficiency syndrome or AIDS-related complex. *N. Engl. J. Med.* 322:1333-1340.