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Research Article

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Deoxyadenosine Triphosphate as a Mediator of Deoxyguanosine Toxicity in Cultured T Lymphoblasts

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Abstract

The mechanism by which 2'-deoxyguanosine is toxic for lymphoid cells is relevant both to the severe cellular immune defect of inherited purine nucleoside phosphorylase (PNP) deficiency and to attempts to exploit PNP inhibitors therapeutically. We have studied the cell cycle and biochemical effects of 2'-deoxyguanosine in human lymphoblasts using the PNP inhibitor 8-amino-guanosine. We show that cytostatic 2'-deoxyguanosine concentrations cause G₁-phase arrest in PNP-inhibited T lymphoblasts, regardless of their hypoxanthine guanine phosphoribosyltransferase status. This effect is identical to that produced by 2'-deoxyadenosine in adenosine deaminase-inhibited T cells. 2'-Deoxyguanosine elevates both the 2'-deoxyguanosine-5'-triphosphate (dGTP) and 2'-deoxyadenosine-5'-triphosphate (dATP) pools; subsequently pyrimidine deoxyribonucleotide pools are depleted. The time course of these biochemical changes indicates that the onset of G₁-phase arrest is related to increase of the dATP rather than the dGTP pool. When dGTP elevation is dissociated from dATP elevation by coincubation with 2'-deoxycytidine, dGTP does not by itself interrupt transit from the G₁ to the S phase. It is proposed that dATP can mediate both 2'-deoxyguanosine and 2'-deoxyadenosine toxicity in T lymphoblasts.

Introduction

Inherited deficiency of the enzyme purine nucleoside phosphorylase (PNP,¹ EC 2.4.2.1) is associated with severely impaired cellular immune function (1–3). In contrast, the more common adenosine deaminase (ADA, EC 3.5.4.4) deficiency causes a combined immune deficiency with marked reduction in both humoral and cellular immunity (4, 5). PNP mediates the catabolism of the purine nucleosides guanosine and inosine, and their corresponding deoxynucleosides to the parent bases guanine and hypoxanthine. These bases may then either be salvaged by hypoxanthine guanine phosphoribosyltransferase (HGPRTase) to ribonucleotides or further catabolized to uric acid. All four PNP precursors have been shown to be present at high levels in the

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1. Abbreviations used in this paper: ADA, adenosine deaminase; EBV, Epstein-Barr virus; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; HGPRTase, hypoxanthine guanine phosphoribosyltransferase; (d)NTP, (deoxy)ribonucleotide triphosphate; PNP, purine nucleoside phosphorylase.

urine of PNP-deficient individuals, with elevation of plasma inosine and guanosine and mild hypouricemia (3).

Purine deoxyribonucleosides have long been known to be toxic to lymphoid cells, particularly to those of thymic origin (6). Several mechanisms of 2'-deoxyadenosine toxicity have been proposed and investigated, using ADA inhibitors, such as erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) and deoxycoformycin, which allow biochemical modeling of ADA deficiency (7, 8). Clinical trials of deoxycoformycin as an antilymphoid agent in leukemia and lymphoma have also been reported (9, 10).

The demonstration of elevated levels of dATP in the erythrocytes of ADA-deficient individuals focussed attention on this deoxynucleotide as a mediator of 2'-deoxyadenosine lymphotoxicity (11). This is attractive because T cells have a greater capacity than B cells to elevate their 2'-deoxyadenosine-5'-triphosphate (dATP) levels, providing an explanation for their greater sensitivity to 2'-deoxyadenosine (7, 12–14).

It has long been proposed that the basic mechanism of dATP toxicity in proliferating cells is by inhibiting ribonucleotide reductase (E.C. 1.17.4.1), thus depriving DNA synthesis of substrates such as 2'-cytosine-5'-triphosphate (dCTP)—the “ribonucleotide reductase hypothesis.” This has been supported by studies of mutant murine and human T cells with reduced sensitivity to 2'-deoxyadenosine and dATP-resistant ribonucleotide reductase (15). However, doubt has been cast on this interpretation by the lack of a consistent relationship between deoxynucleotide triphosphate (dNTP) pool changes, primary inhibition of DNA synthesis and end points such as growth inhibition or cell death (6). Further, a study of the differential incorporation of thymidine and uridine into DNA, although supporting a primary inhibition of DNA synthesis, was not consistent with ribonucleotide reductase inhibition (16). Finally, neither the arrest in the G₁ phase of the cell cycle by dividing T lymphoblasts nor the cytotoxicity for resting lymphocytes of micromolar 2'-deoxyadenosine concentrations (in the presence of ADA inhibitor) are explained by current models of inhibition of ribonucleotide reductase (17, 18). Alternative modes of toxicity have been proposed and documented such as inhibition of methylation reactions, ATP depletion, and interference with RNA synthesis (6). Though NAD depletion has now been convincingly associated with 2'-deoxyadenosine-induced cell death in resting lymphocytes (19), at this stage it appears no single mechanism explains all observations in dividing cells.

Earlier studies had also demonstrated the lymphotoxicity of 2'-deoxyguanosine, attention focussing on 2'-deoxyguanosine-5'-triphosphate (dGTP) as a toxic metabolite by virtue of both its capacity to inhibit CDP reduction (and therefore DNA synthesis) and its selective accumulation in T cells (20–23). Elevated dGTP levels were subsequently reported in erythrocytes from a PNP-deficient patient (24). 2'-Deoxyguanosine-resistant mutant mouse lymphoblasts with PNP deficiency and altered ribonucleotide reductase have also been described (25, 26). Nucleoside

incorporation studies have confirmed that DNA synthesis can be inhibited by micromolar concentrations of 2'-deoxyguanosine in the absence of a PNP inhibitor, and have shown that incorporation of uridine into DNA is suppressed more than that of thymidine, consistent with inhibition of ribonucleotide reductase (16).

The recent availability of a reasonably potent PNP inhibitor, 8-aminoguanosine (K_i , 17 μM), has allowed a more refined approach to the pathophysiology of this PNP deficiency and to the evaluation of PNP inhibition as a possible cytotoxic strategy (27). At micromolar concentrations of 2'-deoxyguanosine in the presence of PNP inhibitor, dGTP pool elevation has been documented in T lymphoblasts and mitogen-stimulated lymphocytes, and has been correlated with inhibition of proliferation (28). In contrast, GTP has been shown to be the major toxic metabolite for B lymphoblasts and mature T cell lines (29).

We have examined the effect of 2'-deoxyguanosine on the cell cycle kinetics of PNP-inhibited human T lymphoblasts by flow cytometric techniques. We have correlated these effects with changes in nucleotide pools, comparing and contrasting them with the analogous effects of 2'-deoxyadenosine. We show that, in the presence of a PNP inhibitor, 2'-deoxyguanosine, as well as causing an elevation of the dGTP pool, induces a secondary but delayed rise in the dATP pool, apparently via allosteric effects on ribonucleotide reductase. This secondary dATP rise is associated with the development of G_1 phase arrest, a phenomenon independent of inhibition of ribonucleotide reductase per se.

Methods

Cell lines. Cultured human leukemic lymphoblasts CCRF-CEM and MOLT-4, derived from patients with T cell acute lymphoblastic leukemia were kindly provided by Dr. J. Minowada (Roswell Park Memorial Institute, Buffalo, NY) and the HGPRTase-deficient T lymphoblasts CCRF-CEM-HGPRT₁ and CCRF-CEM-AG₁ by Dr. B. Ullman (Department of Medicine, University of California, San Francisco) and Dr. M. Hershfeld (Department of Medicine and Biochemistry, Duke University Medical Center, Durham, NC) respectively. Epstein-Barr virus (EBV)-transformed B lymphoblasts derived from normal individuals (JP and WIL-2) were from Dr. I. Jack (Royal Children's Hospital, Melbourne, Victoria, Australia). These cell lines had similar doubling times (~ 24 h) and were studied in the logarithmic phase of growth.

Human peripheral blood lymphocytes. Peripheral blood lymphocytes (PBL) were obtained from healthy blood donors. Mononuclear cell suspensions were obtained after Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation, with subsequent monocyte depletion in 150-cm² polystyrene flasks (37°C, 2 h). Cells were then incubated overnight at $1 \times 10^6/\text{ml}$ prior to further experimentation.

Incubation in nucleosides. All cells were maintained at 37°C in suspension culture in HEPES-buffered Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (5 U/ml), and streptomycin (5 $\mu\text{g}/\text{ml}$).

Incubations for growth studies were done in 2-ml multiwell plates (Flow Laboratories, Inc., McLean, VA), while flow cytometry and nucleotide studies used cells grown in polystyrene flasks (Corning Glass Works, Corning, NY). Nucleosides were added to cultures at a density of $2 \times 10^5/\text{ml}$, 8-aminoguanosine and EHNA being added 20–30 min before other nucleosides.

Cell counts (a minimum of 300 cells) were performed manually using a hemocytometer, and viable cells were distinguished from dead cells by phase-contrast microscopy and trypan blue exclusion.

Reagents. Tritiated dNTPs were obtained from the Amersham International (Sydney, Australia). Unlabeled dNTPs and deoxyribonucleosides were obtained from Sigma Chemical Co. (St. Louis, MO). Micro-

coccus luteus DNA polymerase, poly (dA-dT), and poly (dI-dC) were purchased from Miles Laboratories Inc. (Ekhart, IN). 8-Aminoguanosine and EHNA were gifts from Dr. G. Elion and Dr. C. Nichol, respectively, both of Burroughs Wellcome Ltd. (Research Triangle Park, NC). 8-Aminoguanosine was also obtained as a gift from Dr. L. B. Townsend of the School of Chemistry, University of Michigan (Ann Arbor, MI). Hoechst 33342 was obtained from Calbiochem-Behring Corp. (Sydney, Australia). All other chemicals were of analytical grade.

Flow cytometry. Cellular DNA content was measured using a Phywe ICP 22 mercury lamp based flow cytometer (Ortho Instruments, Westwood, MA). Routine DNA staining was performed by addition of 300 μl of ethidium bromide (400 $\mu\text{g}/\text{ml}$ in 1% Triton X-100) and 300 μl of mithramycin (125 $\mu\text{g}/\text{ml}$ in 1% Triton X-100, 75 mM MgCl₂) to 10⁶ cells in 2 ml of complete medium. Samples of the stained cells were then excited at 360–460 nm and the resulting fluorescence was measured at wavelengths below 550 nm (30).

For the 5-bromodeoxyuridine labeling experiment, DNA staining with Hoechst 33342 was performed as previously described (30, 31). Briefly, 10⁶ cells in 2 ml of complete medium were permeabilized by addition of 1.5 vol of ice-cold 0.1% Triton X-100 containing 0.1 M HCl and 0.15 M NaCl. After 2 min the cells were centrifuged and the pellet was resuspended in 0.1 M Tris-HCl buffer, pH 7.4, containing Hoechst 33342 (1 $\mu\text{g}/\text{ml}$). Samples of stained cells were then excited at 360 nm and the resulting fluorescence measured at 450 nm (450±10 nm bandpass filter).

Approximately 30,000 cells were analyzed for each DNA content histogram. Unfixed chicken erythrocytes (10⁶) were added to each sample before staining as an internal standard to allow for any variation in machine performance and staining procedures. Calculations of percentages of cells in various phases of the cell cycle were made using a curve-fitting method of analysis (32). Typically, aliquots taken simultaneously from the same culture and separately stained for DNA content as described above yielded values for cell-cycle phase percentages that varied from the mean by standard deviations of less than 1%. For example, a logarithmically growing culture of CCRF-CEM cells (sampled in triplicate with five separate analyses per aliquot stained) gave the following results (mean±SD): % G_1 = 42.1±0.4, %S = 44.4±0.2, % G_2/M = 13.2±0.4.

Nucleotide (dNTP and NTP) analysis. Aliquots of 5–10 $\times 10^6$ cells were spun and washed once in Dulbecco's phosphate-buffered saline (in g/100 ml: NaCl 0.8, KCl 0.02, Na₂HPO₄ 0.115, KH₂PO₄ 0.02) containing EDTA 2 mM, extracted in ice-cold 60% ethanol and stored at -20°C. After lyophilization, cell extracts were taken up in 500 μl of 10 mM Tris buffer, pH 7.85, and spun at 40,000 g at 4°C for 10 min and the supernatants were stored at -20°C.

dNTP pools were measured by a modification of the DNA polymerase assay (17, 33, 34). 50- μl aliquots of the supernatants, or appropriate standard solutions of dNTP, were assayed in duplicate in a reaction mixture containing 0.75 mM MgCl₂, 40 mM Tris buffer, pH 7.85, 2 mM dithiothreitol, and 0.2 μl of DNA polymerase in a total volume of 200 μl . dTTP and dATP pools were assayed using a poly (dA-dT) template and [³H]dATP and [³H]dTTP, respectively (0.2 $\mu\text{Ci}/\mu\text{mol}/\text{assay}$). dGTP and dCTP were assayed using a poly (dI-dC) template and [³H]dCTP and [³H]dGTP, respectively (0.2 $\mu\text{Ci}/\mu\text{mol}/\text{assay}$). After a 60-min incubation at 37°C, the reaction was stopped with 0.1 ml of 0.04 M sodium pyrophosphate. The trichloroacetic acid precipitates were filtered and the dried filter papers were counted in organic counting scintillant (Amersham International) in an LKB1215 Rack-Beta scintillation counter (Wallac Oy, Turku, Finland). Measured dNTP values were corrected for isotope dilution by complementary dNTPs from the cell extracts.

Ribonucleotide triphosphates (NTPs) were quantified by high-performance liquid chromatography as described (29). NTPs and dNTPs were eluted from a Partisil-10 SAX anion exchange column (Whatman Laboratory Products, Inc., Clifton, NJ) using a linear ammonium phosphate gradient (0.30–0.45 M, pH 3.3–3.6 over 35 min) at a flow rate of 2 ml/min. Nucleotides were detected and identified by their absorbances at 254 and 280 nm and compared with calibration curves of nanomole amounts of pure standards.

Results

Growth inhibition of lymphoblasts by 2'-deoxyguanosine. The effect of 2'-deoxyguanosine and coincubation with 8-aminoguanosine on the growth of wild-type and HGPRTase-deficient T lymphoblasts is shown in Table I. Concentrations of up to 500 μM 8-aminoguanosine were studied and, though it did not inhibit growth by itself, 8-aminoguanosine potentiated the toxicity of 2'-deoxyguanosine in a concentration-dependent fashion. 50 μM 8-aminoguanosine caused more than a fourfold reduction in the ID_{50} (48 h) of 2'-deoxyguanosine with no significant further potentiation at higher concentrations. Further experimentation focused on conditions that caused "cytostasis," by which we mean maximal inhibition of growth without significant reduction of cell numbers from initial control values. In the T lymphoblasts studied, this criterion was met at 20 μM 2'-deoxyguanosine in the absence of 8-aminoguanosine and at 5 μM 2'-deoxyguanosine in its presence, though small (<5%) percentages of nonviable cells could be detected at slightly lower concentrations of 2'-deoxyguanosine. Conditions that did lead to net cell killing have been designated "cytoidal."

The EBV-transformed B lymphoblasts studied (Table I) had somewhat lower sensitivities to 2'-deoxyguanosine than T cells. 2'-Deoxyguanosine toxicity was not enhanced by 50 μM 8-aminoguanosine and, as recently reported (29), increasing the 8-aminoguanosine concentration was protective: the ID_{50} for JP lymphoblasts rose to 60 μM if 8-aminoguanosine was increased to 250 μM .

Analytical DNA flow cytometric analysis of 2'-deoxyguanosine growth inhibition. A cytostatic concentration of 2'-deoxyguanosine (20 μM) alone produced a cell cycle-nonspecific block in wild-type T lymphoblasts (Fig. 1 B), whereas HGPRTase-deficient T lymphoblasts were predominantly blocked in the G_1 phase of the cell cycle (Fig. 1 C). Though unexpected, this blockade was similar to that induced by 2'-deoxyadenosine in

Table I. Effects of 2'-Deoxyguanosine and 8-Aminoguanosine on the Growth of Lymphoblasts

Cell line	ID_{50} 2'-deoxyguanosine	
	-8-AGuo	+8-AGuo
	μM	μM
T lymphoblasts		
CCRF-CEM	14	3
MOLT-4	14	3
CCRF-CEM-HGPRT ₁ (HGPRTase-deficient)	16	4
B lymphoblasts		
JP	19	16
WIL-2	32	30

The lymphoblasts shown were cultured from an initial concentration of 2.0×10^5 cells/ml in RPMI 1640 medium plus 10% fetal bovine serum. 2'-Deoxyguanosine was added at increasing concentrations in either the presence or the absence of 50 μM 8-aminoguanosine (8-AGuo). Viable cell counts were performed at 48 h, expressed as a percentage of counts in control cultures and the 2'-deoxyguanosine concentration causing 50% growth inhibition (ID_{50}) was determined graphically. Values given are the means of duplicate determinations performed on at least two occasions.

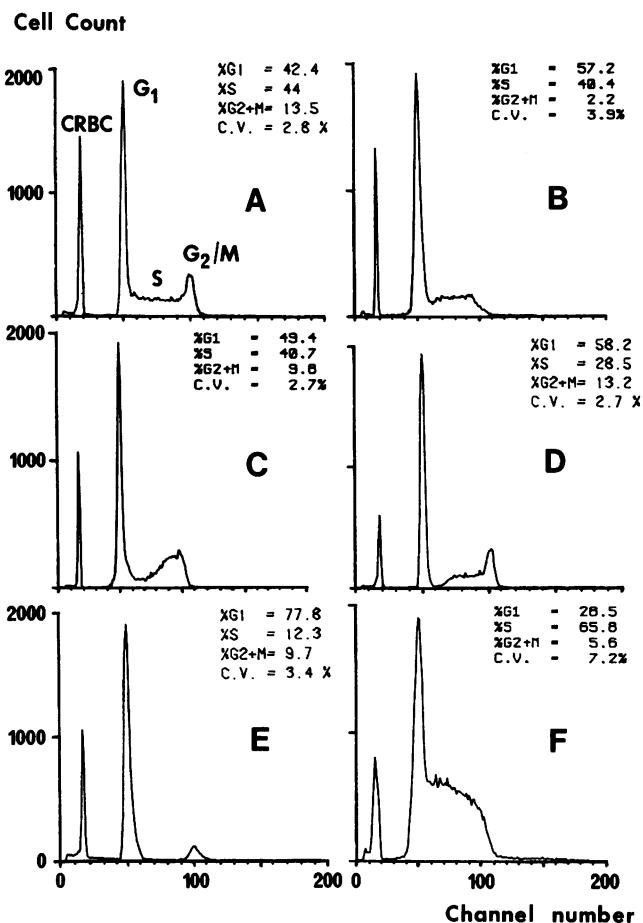


Figure 1. Effects of cytostatic concentrations of 2'-deoxyguanosine (dGuo) and 8-aminoguanosine (8-AGuo) on cell cycle kinetics. T lymphoblasts (CCRF-CEM), EBV-transformed B lymphoblasts (JP), and HGPRTase-deficient T lymphoblasts (CCRF-CEM-HGPRT₁) cultured in separate experiments as described in Table I were incubated in dGuo and 8-AGuo at the concentrations shown. At the times indicated aliquots were stained with ethidium bromide and mithramycin and flow cytometric DNA microfluorimetry was performed as described in Methods. Histograms are shown in the order discussed in Results and are representative of numerous independent experiments on each cell type. CRBC, chicken erythrocyte internal standard; C.V., coefficient of variation. (A) CCRF-CEM: representative normal histogram. (B) CCRF-CEM: dGuo 20 μM only, 24 h. (C) CCRF-CEM-HGPRT₁: dGuo 20 μM only, 6 h. (D) CCRF-CEM: dGuo 5 μM + 8-AGuo 50 μM , 6 h. (E) CCRF-CEM: dGuo 5 μM + 8-AGuo 50 μM , 24 h. (F) JP: dGuo 20 μM + 8-AGuo 50 μM , 24 h.

ADA-inhibited T lymphoblasts (17). This indicated that the specific G_1 -phase arrest seen in HGPRTase-deficient T cells was due to residual 2'-deoxyguanosine, its deoxyribonucleotides, or direct products of its hydrolysis by PNP and not to guanine salvage. We therefore proceeded to examine the effect of PNP inhibition on 2'-deoxyguanosine toxicity in wild-type T lymphoblasts.

In the presence of the PNP inhibitor 8-aminoguanosine, a cytostatic concentration of 2'-deoxyguanosine (5 μM) caused a specific G_1 -phase arrest, leading to a maximum of 80–90% G_1 cells by 18 h (Fig. 1, D and E). If no further 2'-deoxyguanosine was added, these cells moved into S phase and recovered over the subsequent 24–48 h. Early, mid-, and late S phase had been

depleted sequentially, implying that cells already synthesizing DNA were able to complete that S phase, pass through G₂ and mitosis, and return to G₁ where they were blocked. 5'-bromodeoxyuridine suppression of Hoechst 33342 fluorescence (Fig. 2) confirmed this interpretation, as was the case in the G₁ block caused by cytostatic concentrations of 2'-deoxyadenosine in ADA-inhibited T lymphoblasts (31).

DNA that has incorporated 5-bromouracil instead of thymine binds the fluorochrome combination ethidium bromide/mithramycin normally but does not bind Hoechst 33342, whereas normal DNA binds both stains quantitatively. Hoechst 33342 DNA microfluorimetry of cultures incubated in 5-bromodeoxyuridine alone thus shows progressive diminution of the original G₁ peak as cells, having been exposed to 5-bromodeoxyuridine for increasing periods of time during S phase, return to form a second, less fluorescent G₁ peak (Fig. 2 B). CCRF-CEM T lymphoblasts were incubated in the presence of 5 μ M 5-bromodeoxyuridine, 5 μ M 2'-deoxyguanosine, and 50 μ M 8-aminoguanosine, and aliquots were taken at 6-h intervals for Hoechst 33342 DNA microfluorimetry. This concentration of 5-bromodeoxyuridine does not perturb the growth or cell cycle kinetics of these cells. We noted a G₁ block, sequential depletion of S phase, and the accumulation of a diffuse, less fluorescent second G₁ peak representing diploid cells that had incorporated 5-bromodeoxyuridine into DNA (Fig. 2 D). This confirmed our impression of a differential effect of 2'-deoxyguanosine in the presence of PNP inhibitor, namely that, whereas G₁ cells were

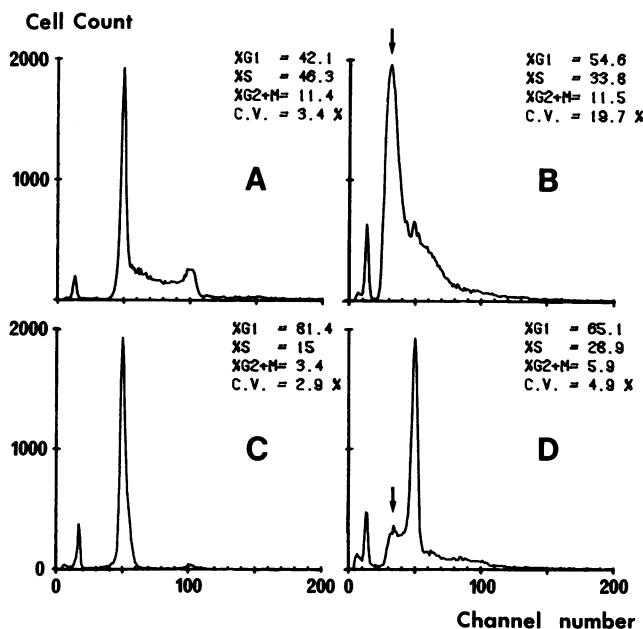


Figure 2. Effects of 2'-deoxyguanosine (dGuo) and 8-aminoguanosine (8-AGuo) on cell cycle kinetics: 5-bromodeoxyuridine (BrdUrd) suppression of Hoechst 33342 fluorescence. CCRF-CEM T lymphoblasts cultured as described in Table I with the additives indicated were stained with Hoechst 33342 and flow cytometric DNA microfluorimetry was performed (as described in Methods) after the times stated. (A) No additives, 0 h. (B) BrdUrd 5 μ M, 24 h. (C) dGuo 5 μ M + 8-AGuo 50 μ M, 24 h. (D) dGuo 5 μ M + 8-AGuo 50 μ M + BrdUrd 5 μ M, 24 h. Positions of CRBC standard and fully stained G₁ and G₂/M peaks are as shown in Fig. 1 A. Arrows indicate cells that have incorporated BrdUrd.

unable to enter S phase, DNA synthesis by cells already in S phase proceeded to completion unhindered.

PNP-inhibited T lymphoblasts incubated at 20 μ M 2'-deoxyguanosine (a cytotoxic concentration) showed nonspecific cell cycle changes. Initially there was some accumulation of cells in G₁ but, over time, neither increase nor depletion of cells in S or G₂/M phases. In the context of complete growth arrest prior to cell killing this nonspecific cell cycle block indicated impairment of entry into S phase, of ongoing DNA synthesis, and possibly of mitosis as well. PNP-inhibited HGPRTase-deficient T lymphoblasts showed cell-cycle changes identical to those induced in PNP-inhibited wild-type cells at both cytostatic and cytotoxic 2'-deoxyguanosine concentrations (results not shown). In contrast, B lymphoblasts at a cytostatic 2'-deoxyguanosine concentration (20 μ M) showed predominantly S-phase blockade whether or not PNP inhibitor was present (Fig. 1 F).

All these observations were replicated in further human T

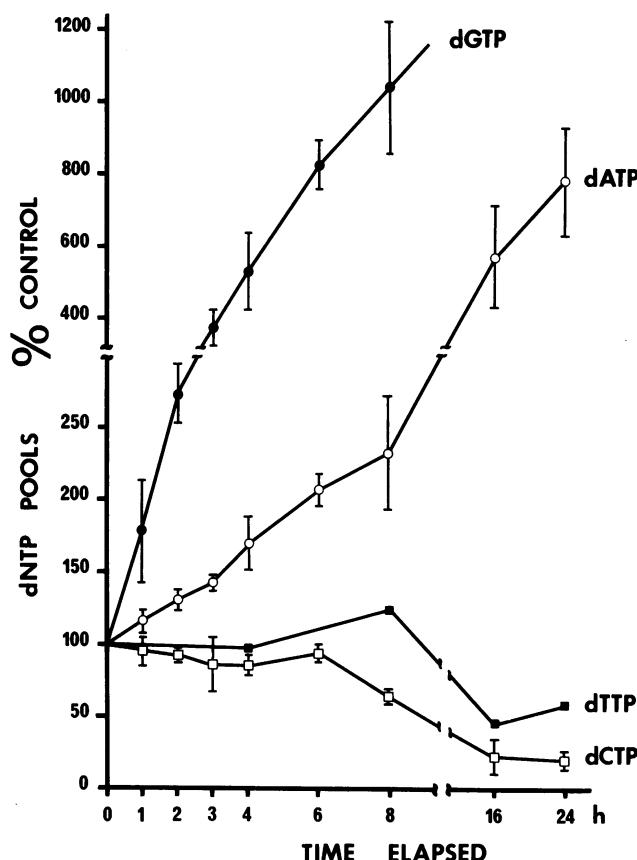


Figure 3. Effect of cytostatic concentration of 2'-deoxyguanosine (dGuo) and 8-aminoguanosine (8-AGuo) on dNTP pools in CCRF-CEM lymphoblasts. Cells were cultured as described in Table I. At the times indicated after commencing incubation with 5 μ M dGuo + 50 μ M 8-AGuo, aliquots of 5×10^6 cells were removed, washed, and extracted with 60% ethanol. Aliquots of reconstituted lyophilized extract were assayed in duplicate for dNTP pools by the DNA polymerase method (as described in Methods). Values shown for dGTP, dATP, and dCTP pools are expressed as percentage of control values and up to 8 h represent mean \pm SD from at least five experiments, while all other results show mean \pm SD from at least two experiments. Control values (pmol/10⁶ cells): dGTP 25.0 \pm 3.0, dATP 40.2 \pm 8.6, dCTP 22.5 \pm 2.7, dTTP 48.5 \pm 11.3. (●) dGTP; (○) dATP; (■) dTTP; (□) dCTP.

lymphoblast, HGPRTase-deficient T lymphoblast and B lymphoblast lines (MOLT-4, CCRF-CEM-AG₁, and WIL-2, respectively) (results not shown).

Effect of 2'-deoxyguanosine on nucleotide pools. 8-aminoguanosine did not by itself significantly alter dNTP or GTP levels. In PNP-inhibited T lymphoblasts 5 μ M 2'-deoxyguanosine perturbed all four dNTP pools, with prompt elevation of both dGTP and dATP and subsequent delayed depletion of dCTP and to a lesser extent dTTP (Fig. 3). This pattern was consistent with stimulation by dGTP of ADP reduction and inhibition of CDP and UDP reduction according to the standard model of allosteric regulation of ribonucleotide reductase (34–36).

Inasmuch as the same cell-cycle perturbation, G₁-phase arrest, can be produced by either 2'-deoxyadenosine or 2'-deoxyguanosine in T lymphoblasts, it is therefore significant that dATP elevation is common to both situations. In 2'-deoxyadenosine-treated cells it is the only early dNTP abnormality (17), whereas in cells treated with 2'-deoxyguanosine, dATP elevation is associated with but preceded by dGTP elevation. Because it seemed that the cause of the G₁-phase arrest would be most evident in the biochemical changes pertaining at the time of its onset (rather than hours or days later), the time courses of the various dNTP changes were compared more closely.

ADA-inhibited CCRF-CEM T lymphoblasts exposed to 3 μ M 2'-deoxyadenosine began to accumulate excess cells in G₁ from 2 h (Fig. 4A), by which time the dATP pool had increased by 1.5-fold (Fig. 4B). dATP continued to rise rapidly whereas the other dNTPs were unchanged until 8 h had elapsed, as has

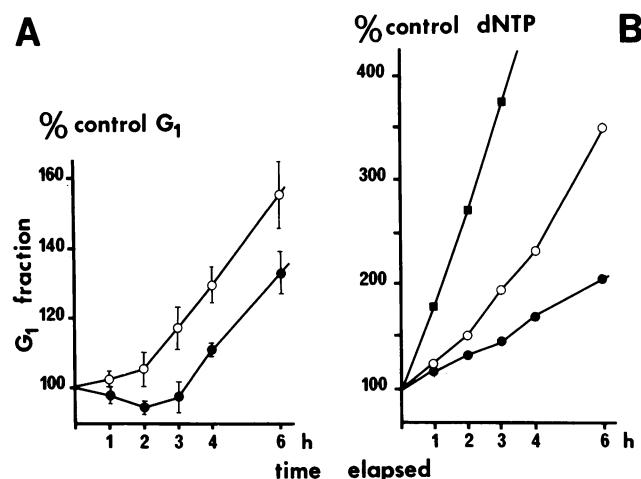


Figure 4. Relationship of time of onset of G₁-phase arrest to changes in dNTP pools of either 2'-deoxyguanosine (dGuo) or 2'-deoxyadenosine (dAdo)-treated T lymphoblasts. CCRF-CEM lymphoblasts in parallel experiments were incubated in either dGuo 5 μ M plus 8-aminoguanosine (8-AGuo) 50 μ M (closed symbols) or dAdo 3 μ M plus EHNA 5 μ M (open symbols). At the times indicated aliquots were taken for DNA microfluorimetry and dNTP pools analysis as described in Methods. (A) The proportion of cells in G₁ phase of the cell cycle is expressed as a percentage of the initial G₁ fraction in that experiment (see Methods). Values shown are mean \pm SD from at least four experiments with each nucleoside combination. (○) dAdo + EHNA experiments; (●) dGuo + 8-AGuo experiments. (B) dATP and dGTP pools are expressed as in Fig. 3 and are mean values from at least four experiments. (○) dATP in dAdo + EHNA experiments; (●) dATP in dGuo + 8-AGuo experiments; (■) dGTP in dGuo + 8-AGuo experiments.

previously been described (17). By 24 h dGTP had fallen to 20% of control whereas dCTP and dTTP had increased by 1.5- and threefold, respectively (data not shown), but no further cell-cycle disturbances were noted. At the time of onset of G₁-phase arrest the only abnormality was an expansion of the dATP pool of about 1.5-fold.

In PNP-inhibited CCRF-CEM lymphoblasts exposed to 5 μ M 2'-deoxyguanosine, the percentage of cells in G₁ phase did not rise until after 3 h of incubation (Fig. 4A). By this time both the dGTP and dATP pools had increased but, whereas the dGTP pool had exceeded control levels by 1.8-fold by 1 h and 3.5-fold by 3 h, the dATP pool rose more gradually, reaching 1.5 times control at about 3 h (Fig. 4B). By 24 h dGTP was 30 times and dATP eight times control (Fig. 3). Thus although 2'-deoxyguanosine induced a dGTP elevation steeper than the analogous dATP rise induced by 2'-deoxyadenosine, G₁-phase arrest in 2'-deoxyguanosine-treated cells was delayed until a dATP increase of about 1.5 times control occurred. This dATP increase was similar to that seen in the 2'-deoxyadenosine-treated cells at the time G₁-phase arrest became apparent. The dCTP pool did not fall significantly until 8 h had elapsed, but fell to 20% of control by 24 h, whereas the dTTP pool was only reduced after 16 h of incubation (Fig. 3). At 24 h GTP levels were not significantly increased (Table II) and ATP levels were unchanged in four experiments.

When PNP-inhibited CCRF-CEM lymphoblasts were incubated at a higher 2'-deoxyguanosine concentration (20 μ M) dGTP elevation was more rapid, exceeding 20 times control after 6 h. dCTP depletion was similarly rapid and profound (20% of control at 6 h) and dATP elevation was similar to that produced by 5 μ M 2'-deoxyguanosine (data not shown). GTP levels doubled over 24 h (Table II) and ATP levels fell by 47% in two experiments. If 8-aminoguanosine was omitted, 20 μ M 2'-deoxyguanosine caused a 5.6-fold accumulation of GTP in 24 h and dNTP pools changes were one half as marked as when 8-aminoguanosine was present (data not shown).

PNP-inhibited HGPRTase-deficient T lymphoblasts (CCRF-CEM-HGPRT₁) elevated dGTP and dATP pools to 15 times and nine times control after 24 h of incubation in 5 μ M 2'-deoxyguanosine. This contrasted with the reported (29) inability of HGPRTase-deficient B lymphoblasts to elevate dGTP in the same way and further confirms that the particular sensi-

Table II. Effect of 2'-Deoxyguanosine on GTP Levels in PNP-inhibited T Lymphoblasts

Additives	GTP levels	
	-dCyd	+dCyd
	% control	% control
None (8-AGuo alone)	93 \pm 16 (3)	—
dGuo 5 μ M	124 \pm 50 (4)	97 \pm 28 (2)
dGuo 20 μ M	239 \pm 66 (2)	674 \pm 141 (2)

CCRF-CEM T lymphoblasts were incubated in 50 μ M 8-aminoguanosine (8-AGuo), 2'-deoxyguanosine (dGuo), and either the presence or absence of 10 μ M 2'-deoxycytidine (dCyd). After 24 h aliquots of 5–10 \times 10⁶ cells were extracted in 60% ethanol and GTP levels were measured by high-pressure liquid chromatography as described in Methods. Values are expressed as percentage of control \pm SD. (no. of experiments). Control GTP levels were 535 \pm 116 pmol/10⁶ cells (four experiments).

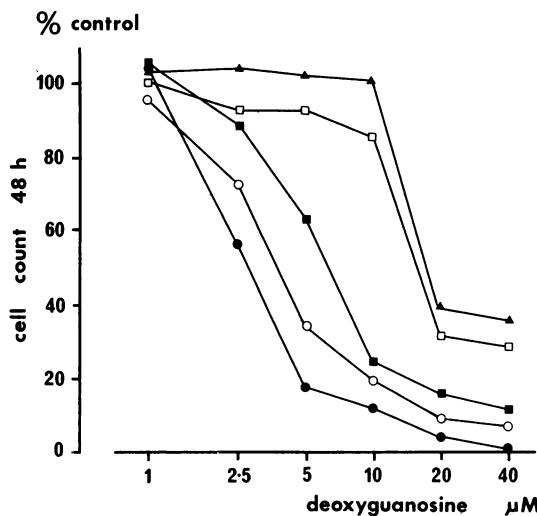


Figure 5. Effects of varying concentrations of 2'-deoxycytidine and 2'-deoxyguanosine on the growth of PNP-inhibited T lymphoblasts. CCRF-CEM lymphoblasts were incubated in the presence of 50 μ M 8-aminoguanosine and varying concentrations of 2'-deoxyguanosine and 2'-deoxycytidine. Viable cell counts were determined after 48 h and expressed as a percentage of control cell counts at the same time. 2'-deoxycytidine concentrations: (●) 0 μ M; (○) 0.3 μ M; (■) 1.0 μ M; (□) 3.0 μ M; (▲) 10 μ M.

tivity of T lymphoblasts to 2'-deoxyguanosine is associated with the capacity of the T phenotype for accumulation of deoxyribonucleotides. B lymphoblasts (JP), as previously reported (29), failed to elevate their dGTP or dATP pools significantly.

Effect of coincubation with 2'-deoxycytidine. 2'-Deoxycytidine wholly or partially prevents the cytotoxic effects of 2'-deoxyadenosine/ADA inhibitor combinations on T lymphoblasts, apparently by competing with 2'-deoxyadenosine for phosphorylation (17). At low concentrations of 2'-deoxyguanosine (10 μ M or less) in the presence of 8-aminoguanosine, 2'-deoxycytidine protected T lymphoblasts from growth inhibition in a concentration-dependent manner, such that normal growth was maintained by 10 μ M 2'-deoxycytidine. At higher 2'-deoxyguanosine concentrations (up to 40 μ M was tested) protection was partial, but cell counts did not fall provided at least 3 μ M 2'-deoxycytidine was present (Fig. 5).

At 5 μ M 2'-deoxyguanosine in the presence of 8-aminoguanosine, coincubation with 2'-deoxycytidine prevented G₁-phase arrest (Fig. 6, A and B). After 24 h the dGTP pool had doubled and the dCTP and dTTP pools were respectively 1.3 and 1.7 times control values, but dATP had not increased (Table III).

GTP levels were unchanged (Table II) while ATP levels fell by 16% in two experiments.

At 20 μ M 2'-deoxyguanosine in the presence of 8-aminoguanosine, coincubation with 2'-deoxycytidine prevented cell death; however growth was not restored (Fig. 5) and DNA microfluorimetry at 24 h showed this was due to a block in S phase of the cell cycle (Fig. 6, C and D). By this time the dGTP pool was 10 times control, dATP and dCTP had fallen to 20% and 80% of control, respectively, and the dTTP pool had increased 1.9-fold (Table III). GTP was elevated over sixfold (Table II) and ATP was reduced to 46% of control in two experiments. Despite these multiple biochemical abnormalities including pronounced dGTP elevation, it is noteworthy that transit from the G₁ to the S phase was completely unimpaired. This especially argues against there being a dGTP-sensitive "target" capable of causing G₁ arrest independently of dATP.

Effect of 2'-deoxyguanosine on peripheral blood lymphocytes. ADA-inhibited PBL are exquisitely sensitive to 2'-deoxyadenosine—only 20% survive 96 h of incubation in 1 μ M 2'-deoxyadenosine, having accumulated dATP (18). In contrast, PBL were only killed by 2'-deoxyguanosine concentrations of at least 100 μ M, the presence or absence of 50 μ M 8-aminoguanosine having little effect (Fig. 7). After 24 h at 300 μ M 2'-deoxyguanosine, the dGTP pool in PNP-inhibited cells was elevated to over 10 times control, but no dATP rise was detected. The latter was not surprising, given the fact that recorded activities of ribonucleotide reductase in PBL are very low (37), and gives indirect support to the notion that 2'-deoxyguanosine elevates the dATP pool in proliferating lymphoid cells by stimulation of ADP reduction.

Discussion

We have shown that, at cytostatic concentrations of 2'-deoxyguanosine, PNP-inhibited T lymphoblasts committed to DNA synthesis complete that cycle and proceed through G₂ and mitosis, whereas cells already in G₁ or returning there are arrested. T lymphoblasts incubated at cytostatic concentrations of 2'-deoxyadenosine and an ADA inhibitor differ only in that they manifest an earlier onset of G₁-phase arrest. Comparing the respective elevations of dGTP and dATP in these experiments, it is apparent that the time of onset of the G₁ block correlates best with expansion of the dATP pool rather than with the other dNTP pool perturbations. Further, when 2'-deoxycytidine protects these cells from G₁-phase arrest dATP is not elevated but dGTP is. Higher, cell-killing 2'-deoxyguanosine concentrations cause mixed cell cycle and biochemical effects.

Table III. Effect of 2'-Deoxycytidine on 2'-Deoxyguanosine Toxicity

Additives	dGTP	dATP	dCTP	dTTP	% Control growth at 48 h		Cell-cycle distribution at 24 h
	% control at 24 h						
dGuo 5 μ M	>2,000	505	20	60	17		G ₁ -phase arrest
dGuo 5 μ M + dCyd 10 μ M	235	115	130	170	102		Normal
dGuo 20 μ M	>2,000	355	20	40	4		Nonspecific arrest
dGuo 20 μ M + dCyd 10 μ M	970	20	80	190	29		S-phase arrest

CCRF-CEM T lymphoblasts were incubated in the presence of 50 μ M 8-aminoguanosine and the concentrations of 2'-deoxyguanosine (dGuo) and 2'-deoxycytidine (dCyd) shown. At 24 h aliquots (5×10^6 cells) were taken for dNTP analysis as described in Fig. 3. dNTP pools are expressed as percentage of the control values given in Fig. 3 and are tabulated against growth inhibition at 48 h and cell-cycle phase distribution at 24 h as determined by DNA microfluorimetry (see Figs. 5 and 6). Values for dNTP pools are the means from two experiments.

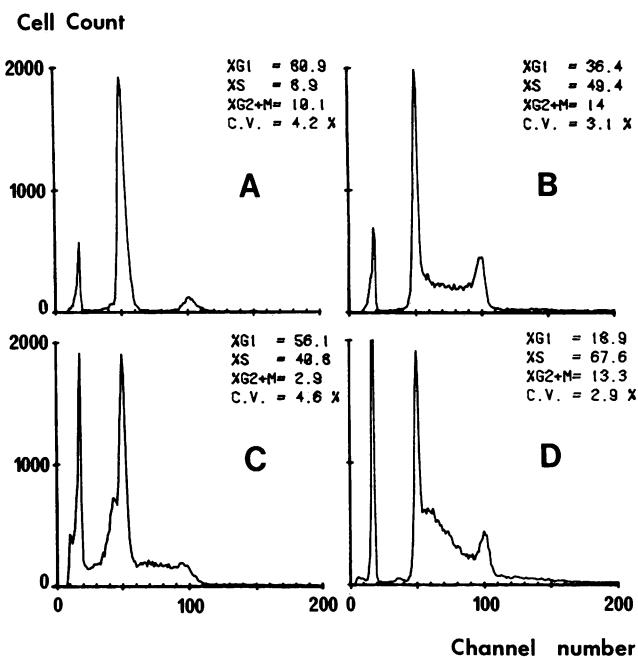


Figure 6. Effect of 2'-deoxycytidine (dCyd) and varying concentrations of 2'-deoxyguanosine (dGuo) on cell-cycle phase distribution of PNP-inhibited T lymphoblasts. CCRF-CEM lymphoblasts were incubated with 50 μ M 8-aminoguanosine and the following additives. (A) dGuo 5 μ M. (B) dGuo 5 μ M + dCyd 10 μ M. (C) dGuo 20 μ M. (D) dGuo 20 μ M + dCyd 10 μ M. After 24 h aliquots were stained with ethidium bromide and mithramycin and DNA content was determined as described in Methods.

Secondary dATP pool elevation has previously been described in 2'-deoxyguanosine-treated human and murine lymphoblasts (16, 21), mitogen-stimulated human lymphocytes (34), and S-phase ("large") human thymocytes (37), but has not drawn comment as a direct mediator of 2'-deoxyguanosine toxicity. It almost certainly occurs by allosteric stimulation of ADP reduction by dGTP; this effect has been demonstrated in ribonucleotide reductase preparations, including that isolated from a human lymphoblastoid line (38).

However, a proposal that dATP is the common mediator by which both 2'-deoxyadenosine and 2'-deoxyguanosine cause G₁-phase arrest must take into account other known modes of 2'-deoxyguanosine toxicity. These are chiefly the ribonucleotide reductase (dNTP depletion) hypothesis and guanine ribonucleotide accumulation and will be dealt with in turn.

dCTP is depleted at cytostatic 2'-deoxyguanosine concentrations but this is quite delayed and may reflect the normal low dNTP pools of G₁ cells as much as inhibition of CDP reductase by dGTP. The ribonucleotide reductase hypothesis would predict interruption of ongoing DNA synthesis by dCTP depletion, but we have shown that cells already in S phase complete DNA synthesis unimpeded. Specific inhibitors of ribonucleotide reductase such as hydroxyurea cause an S-phase block at cytostatic concentrations (17). In further contrast the G₁-phase arrest induced by 2'-deoxyadenosine is associated with a static or slightly elevated dCTP pool. The evidence is thus against a role for dCTP depletion in 2'-deoxyguanosine-induced G₁-phase arrest.

Catabolism of 2'-deoxyguanosine and salvage to guanine ribonucleotides is an important cause of its toxicity to B lymphoblasts and mature T cell lines, but not for HGPRTase-deficient

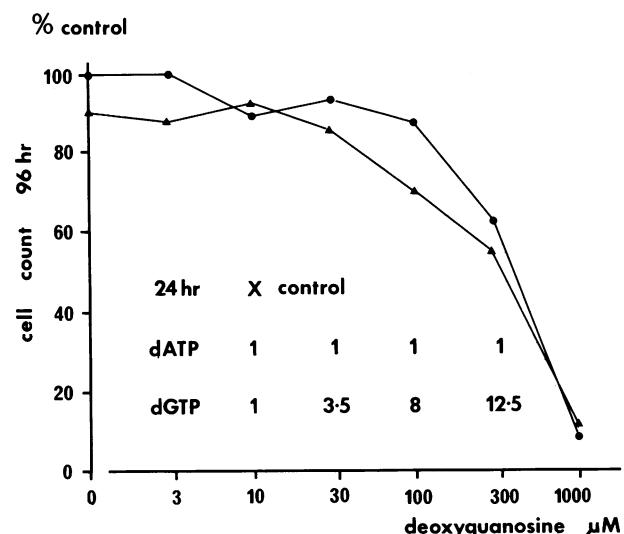


Figure 7. Effect of varying concentrations of 2'-deoxyguanosine (dGuo) and 8-aminoguanosine (8-AGuo) on viability of PBL. PBL were isolated from fresh buffy coats by Ficoll-Paque centrifugation as described in Methods and incubated at 1.0×10^6 /ml in RPMI 1640 + 10% fetal bovine serum. After 24 h dGuo and 8-AGuo were added at the concentrations shown. After a further 96 h viable cell counts were determined and the results were expressed as a percentage of the cell count at 96 h in cultures without additives (which was always at least 85% of the starting count). Values displayed for dATP and dGTP pools were determined after 24 h of incubation in 50 μ M 8-AGuo and dGuo and are expressed as multiples of levels in control cultures (dATP 1.0 ± 0.5 , dGTP 2.0 ± 0.5 pmol/ 10^6 cells) at the same time. Results shown are the mean of two experiments. 8-AGuo concentrations: (●) 0 μ M; (▲) 50 μ M.

B cell lines or PNP-inhibited T lymphoblasts (29, 39). In our experiments with T lymphoblasts GTP was not increased by 2'-deoxyguanosine concentrations that caused G₁-phase arrest, and we have found that HGPRTase-deficient T lymphoblasts similarly block in G₁. At higher concentrations of 2'-deoxyguanosine GTP elevation occurred indicating some escape from the competitive inhibition of PNP by 8-aminoguanosine, an effect described by others (29, 39, 40). This would have contributed to the nonselective cell-cycle changes, such as delay in S or G₂/M, and the cell killing seen under these conditions. The mechanisms of guanine ribonucleotide toxicity are not fully clear as a wide variety of cellular functions may be involved, but they have been shown to include inhibition of de novo purine synthesis (41); this may account for the reduction in ATP caused by high 2'-deoxyguanosine concentrations in our experiments. As is shown by the cell cycle changes in B cells reported by ourselves and others (41), DNA synthesis may also be affected by guanine ribonucleotide accumulation.

Finally, it may be proposed that in 2'-deoxyguanosine-treated T lymphoblasts an as yet unidentified effect of dGTP causes the G₁ arrest despite the concurrent dATP increase. Dissociation of dGTP elevation from dATP elevation is difficult in principle given that the latter is caused by the former, but we feel the 2'-deoxycytidine "rescue" experiments do so and make such a role for dGTP unlikely.

As in 2'-deoxyadenosine toxicity, coincubation with 2'-deoxycytidine can wholly prevent 2'-deoxyguanosine toxicity (6, 17). A major mechanism in protection from 2'-deoxyaden-

osine toxicity is competition for phosphorylation by a common deoxyribonucleoside kinase, thus limiting or preventing dATP accumulation. Similar competition with 2'-deoxyguanosine has been proposed because 2'-deoxyguanosine, 2'-deoxyadenosine and 2'-deoxycytidine kinase activity may be represented by a single-protein species (6, 7, 21). At the lower, cytostatic 2'-deoxyguanosine concentration normal growth was restored and G₁ arrest prevented by 2'-deoxycytidine despite a persistently (albeit less) elevated dGTP. This argues against direct toxicity of dGTP elevation per se. More conclusive is the observation that at 20 μ M 2'-deoxyguanosine, in the presence of 2'-deoxycytidine, a 10-fold elevation of dGTP did not hinder transit from the G₁ to the S phase in the absence of dATP elevation (which was suppressed by adenine pool depletion).

In summary, of the diverse biochemical changes caused by incubating T lymphoblasts in 2'-deoxyguanosine and PNP inhibitor, only dATP elevation is consistently associated with G₁-phase arrest. Potential mechanisms by which dATP could produce a specific G₁-phase arrest may be considered as either related or unrelated to ribonucleotide reductase. Examples of the latter include the evidence that 2'-deoxyadenosine/ADA inhibitor combinations can interfere with RNA synthesis, as shown by inhibition of transcription in both peripheral blood lymphocytes and CCRF-CEM cells (42). Also, dAMP residues have been shown to be incorporated from dATP into the terminal position of poly(A)⁺ messenger RNA, possibly affecting its subsequent processing (43). Interference by dATP in the synthesis or consumption of other adenine nucleotides such as NAD, poly(ADP-ribose) and diadenosine 5',5''-P¹,P⁴-tetraphosphate (Ap₄A) may occur (19). These compounds have been shown to be associated with the regulation of cell cycle transitions including the initiation and cessation of DNA synthesis. Their deoxy analogues—2'-dNAD, poly(dADP-ribose), and dideoxyadenosine 5',5''-P¹P⁴-tetraphosphate—have been synthesized in vitro but not yet detected in vivo (44, 45).

Despite these possibilities the characteristics of cultured T cell lines (murine and human) with mutation at the dATP binding site on the M₁ subunit of ribonucleotide reductase must be taken into account (15, 46). These cell lines are resistant to growth inhibition by 2'-deoxyadenosine, implying that ribonucleotide reductase is the target for dATP-mediated toxicity, albeit not by the classical model of inhibition in T cells. A possible interpretation of the effects of deoxynucleosides in vitro (at least in whole cells) is that the effects of 2'-deoxyguanosine (and thymidine) on the dNTP pools of T cells are in accord with existing models of the allosteric control of ribonucleotide reductase. However, the effect of 2'-deoxyadenosine on dNTP pools is not in accord with these models; that is, dCTP pools do not fall after the rise in dATP and a G₁-phase arrest occurs. This suggests that in intact cells the interaction of dATP with the allosteric subunit may differ from that in purified enzyme preparations. The subunit behavior of ribonucleotide reductase is now quite involved and a putative "replitase" complex has been described. Comprising DNA polymerase, topoisomerase, thymidylate synthetase, ribonucleotide reductase, and other enzymes concerned with DNA replication, it is proposed to form from the individual enzyme components at the end of G₁ and to serve to appropriately channel metabolites for DNA replication (47, 48). Ribonucleotide reductase allosteric (M₁) and catalytic (M₂) subunits also appear to be assembled together at the G₁/S interface. Because the state of aggregation of M₁ in vitro can be readily altered by dATP, polymers being formed in its presence (49), we pos-

tulate that 2'-deoxyadenosine and 2'-deoxyguanosine toxicity in replicating T cells could be mediated by the binding of dATP to the allosteric subunit of ribonucleotide reductase. This does not necessarily inhibit the catalytic (M₂) subunit but rather, via conformational change and possibly formation of M₁ polymers, interferes with assembly of "replitase" at the G₁/S interface and results in G₁-phase arrest.

As in 2'-deoxyadenosine toxicity, the cell cycle effect of cytostatic 2'-deoxyguanosine concentrations is quite specific. Previous reports of 2'-deoxyguanosine toxicity in proliferating lymphoid cells have for the most part described the effects of high concentrations which have multiple cell-cycle and biochemical effects. Though 8-aminoguanosine does not ideally model absolute PNP deficiency, its use has allowed investigation of the lymphotoxicity of 2'-deoxyguanosine concentrations likely to be encountered in PNP-deficient or inhibited individuals. As a result a final common pathway for 2'-deoxyguanosine and 2'-deoxyadenosine toxicity is apparent, namely an as yet unidentified dATP-sensitive target crucial to progression into the DNA synthetic phase of the cell cycle.

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