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Differential Metabolism of Guanine Nucleosides by Human Lymphoid Cell Lines (42118)

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Abstract. Deficiency of the enzyme purine nucleoside phosphorylase is associated with a specific depletion of T cells which is presumably mediated by its substrate, 2′-deoxyguanosine. Inhibitors of this enzyme are therefore being developed as potential immunosuppressive agents. We have compared the effects of 8-aminoguanosine, a competitive inhibitor of purine nucleoside phosphorylase, on the metabolism of 2′-deoxyguanosine by human T lymphoblasts, B lymphoblasts, and mature T-cell lines. 8-Aminoguanosine markedly potentiates the accumulation of dGTP in T lymphoblasts, but results in increased GTP levels in B lymphoblasts and mature T cells. GTP accumulation is associated with ATP depletion of a magnitude similar to that seen with an inhibitor of *de novo* purine biosynthesis, but does not result in inhibition of either DNA or RNA synthesis. In contrast, direct inhibition of *de novo* purine biosynthesis sharply decreased the incorporation of [^{3}H]uridine into both DNA and RNA. We conclude that the mechanism of cell damage resulting from prolonged accumulation of GTP appears to involve more than inhibition of *de novo* purine biosynthesis and consequent ATP depletion. Perturbations in guanine nucleotide pools resulting from partial inhibition of purine nucleoside phosphorylase activity *in vivo* could result in cellular toxicity not limited to the target T cell population.

Inherited deficiency of the enzyme purine nucleoside phosphorylase (PNP; EC 2.4.2.1) results in profound and selective depletion of T lymphocytes with maintenance of B lymphocyte function and number (1). The four substrates of PNP, inosine, 2′-deoxyinosine, guanosine, and 2′-deoxyguanosine, are all excreted in large amounts in the urine of affected individuals (2), but the diminution in T cells has been attributed to the selective metabolism of 2′-deoxyguanosine to dGTP in T lymphocytes and/or their precursors. DeoxyGTP is an inhibitor of the enzyme ribonucleotide reductase and is felt to be cytotoxic by causing a depletion of other deoxyribonucleoside triphosphates essential for DNA synthesis (3). The evidence for a selective T cell effect of 2′-deoxyguanosine comes from studies on cultured human and mouse T lymphoblasts, where 2′-deoxyguanosine toxicity correlates directly with dGTP accumulation (4–6).

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The specificity of the PNP deficiency state for T cells has led to considerable interest in the development of PNP inhibitors as immunosuppressive agents. 8-Aminoguanosine, an analog of guanosine with a K_i of 8 μM for the PNP-catalyzed phosphorolysis of inosine (7), has been demonstrated to markedly potentiate both the accumulation of dGTP and the cytotoxicity of 2′-deoxyguanosine for T lymphoblasts (8). We have studied the effects of 8-aminoguanosine on the metabolism of 2′-deoxyguanosine by both Epstein–Barr virus-transformed B lymphocyte cell lines and mature T cell lines derived from patients with cutaneous T cell lymphomas. In the presence of 8-aminoguanosine, 2′-deoxyguanosine is metabolized to GTP, rather than to dGTP, in both cell types and GTP accumulation over a 24-hr period has a marked inhibitory effect on cell growth (9). The mechanism of growth inhibition is not known. We have asked what effect elevations of GTP have on the metabolism and cell cycle of B lymphoblasts and mature T-cell lines in an effort to better understand the effect(s) of GTP on cell growth.

Materials and Methods. *Materials.* 8-Aminoguanosine was obtained from Dr. Leroy

0037-9727/85 $1.50

Townsend, Department of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan. Other chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri.

Cell lines. The human T lymphoblast Molt-4 cell line was obtained from HEM Research, Rockville, Maryland. The B lymphoblast MGL-8 line was originally obtained from Dr. John Littlefield, Johns Hopkins University, Baltimore, Maryland. The HUT-78 T_4^+ cell line was obtained from Dr. Paul Bunn, National Cancer Institute, Bethesda, Maryland. All cell lines were grown in the presence of RPMI-1640 medium with 10% horse serum at 37°C in 5% CO_2.

Nucleotide pool determinations. At the completion of designated incubation periods, 5×10^6 cells were extracted in cold 60% methanol. Ribo- and deoxyribonucleotides were separated by high-pressure liquid chromatography on a Partisil-10 SAX anion exchange column using a gradient from 0.3 to 0.45 *M* ammonium phosphate, pH 3.3 to 3.6, at a flow rate of 2 ml/min. Nucleotides were quantitated by comparing the absorbance of peaks at 254 nm to that given by nanomole amounts of pure standards migrating with identical retention times.

DNA and RNA synthesis. Uridine incorporation into DNA and RNA was measured by adding 1 μCi [^{3}H]uridine to 2 ml of cells incubated at a concentration of 5×10^5/ml 2 hr before the end of the incubation period. The cell pellet was washed and the nucleic acids were extracted in 0.5 *N* cold perchloric acid. The sediment was washed in 10% TCA and the RNA hydrolyzed in 0.3 *N* KOH, as previously reported (10). The supernate containing the RNA and the redissolved sediment containing the DNA were counted in Bray's scintillation fluid.

Cell cycle effects. B lymphoblasts incubated with 100 μM 8-aminoguanosine alone or in the presence of 50 μM 2′-deoxyguanosine were washed twice with cold phosphate buffered saline and stained with a solution containing 75 μM propidium iodide, 10 m*M* sodium chloride, 10 m*M* Tris, 700 μg/liter RNase, and 1 ml/liter NP40, pH 8.0, at 4°C for 45 min. The DNA content was determined on a Coulter Epic V flow cytometer.

Results. Molt-4 T lymphoblasts incubated with 8-aminoguanosine and 2′-deoxyguanosine for 24 hr demonstrated over a 100-fold increase in dGTP, with no significant change in GTP (Table I). In striking contrast, dGTP levels remained low in B lymphoblasts and mature T cells, but GTP levels increased approximately fivefold.

The presumed mechanism of GTP elevations in B and mature T cells in the presence of 2′-deoxyguanosine and an inhibitor of PNP is illustrated in Fig. 1. 8-Aminoguano-

TABLE I. EFFECTS OF 2′-DEOXYGUANOSINE AND 8-AMINOGUANOSINE ON GTP, dGTP, AND ATP POOLS IN CULTURED LYMPHOID CELLS

	8-AGuo			8-AGuo + dGuo		
	GTP	dGTP	ATP	GTP	dGTP	ATP
	(pmole/10^6 cells)					
T lymphoblasts	366 ± 154	17 ± 4	2224 ± 463	420 ± 230 ($P > 0.1$)	2130 ± 1400 ($P < 0.001$)	1689 ± 609 ($P < 0.05$)
B lymphoblasts	1230 ± 374	12 ± 3	5704 ± 1664	6717 ± 2277 ($P < 0.001$)	45 ± 7 ($P < 0.001$)	4731 ± 1370 ($P < 0.05$)
Mature T cell line	855 ± 290	13 ± 8	3962 ± 848	4534 ± 1808 ($P < 0.001$)	54 ± 8 ($P < 0.001$)	2280 ± 1470 ($P < 0.005$)

Note. Cell were incubated in the presence of 100 μM 8-aminoguanosine in the absence or presence of 50 μM 2′-deoxyguanosine for 24 hr and 5×10^6 cells were extracted for nucleotide pool determinations. Values represent the means ± SD of between 8 and 13 separate determinations. Values for GTP, dGTP, and ATP in cells incubated with 100 μM 8-aminoguanosine did not differ from those in control cells of the same cell line incubated in the absence of additives.

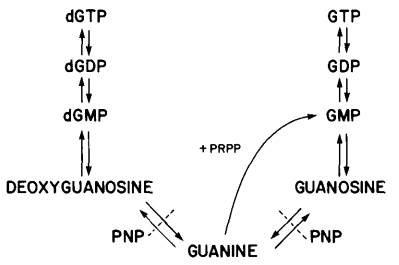

FIG. 1. The metabolism of 2'-deoxyguanosine and guanosine. PNP-purine nucleoside phosphorylase; PRPP-5-phosphoribosyl 1-pyrophosphate. The conversion of guanine to GMP is mediated by hypoxanthine guanine phosphoribosyltransferase activity.

sine is both a weak competitive inhibitor of and a substrate for PNP activity. We have recently demonstrated that this agent will markedly prolong the extracellular $t_{1/2}$ of both guanosine and 2'-deoxyguanosine, but at concentrations of 100 μM will not completely inhibit intracellular PNP activity (9). When the concentration of 8-aminoguanosine is increased to 400 μM, however, it prevents both the accumulation of GTP and the inhibition of cell growth seen at lower concentrations. These data indicate that inhibition of extracellular PNP activity allows the transport of nonphosphorolyzed guanine nucleosides into the cell over a prolonged time period. In the presence of low concentrations of a weak PNP inhibitor, these nucleosides are then cleaved intracellularly by residual PNP activity to guanine and subsequently converted to guanine ribonucleotides by hypoxanthine phosphoribosyltransferase activity (E.C. 2.4.2.8) in the presence of 5-phosphoribosyl 1-pyrophosphate (PRPP). Extracellular guanine, on the other hand, does not increase GTP pools at 24 hr and does not inhibit cell growth.

We then asked whether increased levels of guanine nucleotides caused a depletion of intracellular ATP pools, since GTP is known to have a major negative feedback effect on *de novo* purine biosynthesis. ATP levels decreased in all three cell types in amounts ranging from 17% of control values in the B cells to 42% in the mature T cells. To determine whether ATP depletion indeed resulted from a generalized inhibition of purine biosynthesis, we then compared the time course of ATP depletion in B lymphoblasts with that caused by 2 μM 6-mercaptopurine riboside (6-MMPR), a known inhibitor of *de novo* purine biosynthesis and of cell growth. The plateau in GTP accumulation induced by 8-aminoguanosine plus 2'-deoxyguanosine at 12 hr was accompanied by a nadir in the ATP concentration at the same time point and by a slight decline in UTP pools (Fig. 2A). A similar, but somewhat more profound, reduction in ATP occurred in the cells cultured with 6-MMPR and this was accompanied by a similar drop in GTP pools (Fig. 2B). The UTP pools expanded by a factor of two, a finding which is probably explained by increased *de novo* pyrimidine biosynthesis in the face of reduced GTP levels (11).

We also compared the effects of GTP elevations with those of 6-MMPR on DNA and RNA synthesis in B lymphoblasts (Fig. 3). Over a 24-hr period corresponding to the experiment in Fig. 2, there was no reduction in the incorporation of [^{3}H]uridine into DNA induced by high levels of GTP, and there was an increase in its incorporation into RNA. In striking contrast, 6-MMPR resulted in marked inhibition of [^{3}H]uridine incorporation into both DNA and RNA at 2 hr. The fluctuations in UTP pools and the unknown status of the dTTP pool from which

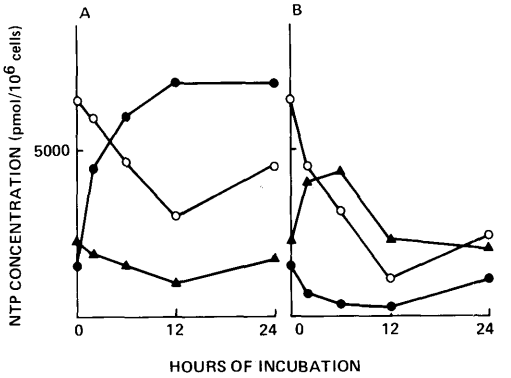

FIG. 2. Comparative effects of 2'-deoxyguanosine and 8-aminoguanosine (A) and 6-mercaptopurine riboside (B) on ribonucleoside triphosphate pools. Cells were incubated in the presence of 50 μM 2'-deoxyguanosine and 100 μM 8-aminoguanosine or 2 μM 6-MMPR. (●) GTP; (○) ATP; (▲) UTP.

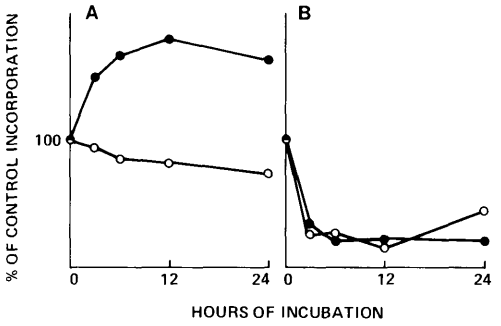

FIG. 3. Comparative effects of 2′-deoxyguanosine and 8-aminoguanosine (A) and 6-mercaptopurine riboside (B) on [^{3}H]uridine incorporation into DNA and RNA. Concentrations of drugs are indicated in the legend to Fig. 2. (○) DNA; (●) RNA.

[^{3}H]uridine would be incorporated into DNA must be taken into account in the interpretation of these isotope incorporation experiments. The data clearly indicate, however, that the elevation of GTP is not markedly inhibitory to either DNA or RNA synthesis at 24 hr and does not produce effects directly analagous to those produced by 6-MMPR.

The effects of GTP elevation on the cell cycle were then examined in B lymphoblasts. 8-Aminoguanosine alone had no effect on the DNA content of these cells when compared to controls (data not shown). The effect of the combination of 8-aminoguanosine plus 2′-deoxyguanosine on the cell cycle at 24 hr is shown in Fig. 4. Sustained elevations of GTP uniformly resulted in marked slowing in the progression of cells through S phase. This effect was also seen in mature T cells. T lymphoblasts accumulating dGTP, on the other hand, were blocked completely at the G_1/S phase boundary.

Discussion. We have demonstrated major differences in the metabolism of 2′-deoxyguanosine between human T lymphoblasts, which accumulate dGTP, and B lymphoblasts and mature T_4^+ cell lines, which accumulate GTP. The marked potentiation of GTP accumulation and inhibition of cell growth by 8-aminoguanosine (9), apparently resulting from the prolonged elevation in extracellular 2′-deoxyguanosine concentrations, has important implications for the development of PNP inhibitors as pharmacologic agents. It has been hoped that the specific defect in mature T cells in PNP-deficiency disease could be mimicked by the administration of PNP inhibitors, thus providing selective immunosuppressive and/or chemotherapeutic drugs. The biochemical rationale for T cell specificity hinges on the selective capability of cultured T lymphoblasts to accumulate dGTP. Our data indicate, however, that incomplete inhibition of PNP activity could result in the widespread accumulation of GTP in other cells, causing nonselective cellular cytotoxicity. In addition, it appears that T_4^+ cells in culture have a markedly different route for guanine nucleoside metabolism than do "immature" T lymphoblasts, making it unlikely that the mature T cell population would be a direct target for toxicity mediated by PNP inhibitors.

We then investigated the mechanism by which GTP might inhibit cell growth. Inhibitors of *de novo* purine biosynthesis are cytotoxic by virtue of depleting the cell of both GTP and ATP. Recent studies in mouse T lymphoma cells have strongly suggested that it is GTP, rather than ATP, depletion which is causally associated with inhibition of DNA synthesis and cell death (12–14). We have confirmed that 6-MMPR depletes B lymphoblasts of both ATP and GTP and markedly inhibits the incorporation of [^{3}H]uridine into DNA and RNA. In contrast, a five-fold elevation in GTP is associated with a 50% depletion in ATP, but no apparent inhibition of either RNA or DNA synthesis. We conclude from these studies that inhibition of *de*

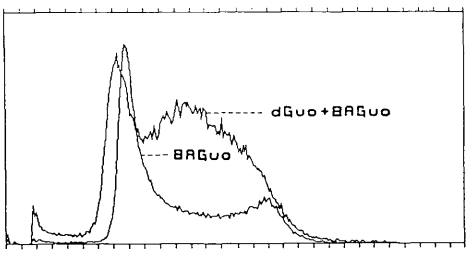

FIG. 4. Effects of 2′-deoxyguanosine and 8-aminoguanosine on the DNA content of B lymphoblasts. Cells were incubated for 24 hr in the presence of 100 μM 8-aminoguanosine in the absence or presence of 50 μM 2′-deoxyguanosine, stained with propidium iodide, and analyzed on a flow cytometer.

novo purine biosynthesis resulting in ATP, but not in GTP, depletion is not sufficient to account for the inhibition of growth.

In a further attempt to define the metabolic effect of GTP elevations, we examined the effects of 8-aminoguanosine plus 2′-deoxyguanosine on the cell cycle of actively proliferating B lymphoblasts. We observed a marked slowing in the progression of the cells through S phase, a relatively nonspecific effect which also has been demonstrated to occur in HL-60 cells exposed to 2′-deoxyguanosine (15). These results do confirm, however, that GTP elevation, in contrast to GTP depletion, does not directly inhibit DNA synthesis. The potential regulatory effects of GTP on cell growth are numerous. GTP-binding proteins are known to regulate the activity of adenylate cyclase (16) and have been related to the growth of normal and malignant cells (17). In addition, the binding of GTP to tubulin appears to be important for normal cellular morphogenesis (18). The definition of the exact role of increased GTP levels in inhibiting lymphocyte growth may not be feasible using metabolic approaches. Nevertheless, definition of this effect has important implications in the potential clinical applications of PNP inhibitors as antilymphocyte agents.

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