

Purinogenic Immunodeficiency Diseases

DIFFERENTIAL EFFECTS OF DEOXYADENOSINE AND DEOXYGUANOSINE ON DNA SYNTHESIS IN HUMAN T LYMPHOBLASTS

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ABSTRACT Deoxyadenosine and deoxyguanosine are toxic to human lymphoid cells in culture and have been implicated in the pathogenesis of the immunodeficiency states associated with adenosine deaminase and purine nucleoside phosphorylase deficiency, respectively. We have studied the relative incorporation of several labeled nucleosides into DNA and into nucleotide pools to further elucidate the mechanism of deoxyribonucleoside toxicity. In the presence of an inhibitor of adenosine deaminase [erythro-9-(2-hydroxy-3-nonyl)adenine [EHNA], 5 μM], deoxyadenosine (1–50 μM) progressively decreased the incorporation of thymidine, uridine, and deoxyuridine into DNA, but did not affect uridine incorporation into RNA. This decrease in DNA synthesis was associated with increasing dATP and decreasing dCTP pools. Likewise, incubation of cells with deoxyguanosine caused an elevation of dGTP, depletion of dCTP, and inhibition of DNA synthesis.

To test the hypothesis that dATP and dGTP accumulation inhibit DNA synthesis by inhibiting the enzyme ribonucleotide reductase, simultaneous rates of incorporation of [^3H]uridine and [^{14}C]thymidine into DNA were measured in the presence of deoxyadenosine plus EHNA or deoxyguanosine, and in the presence of hydroxyurea, a known inhibitor of ribonucleotide reductase. Hydroxyurea (100 μM) and deoxyguanosine (10 μM) decreased the incorporation of [^3H]uridine but not of [^{14}C]thymidine into DNA; both compounds also substantially increased [^3H]cytidine incorporation into the ribonucleotide pool while

reducing incorporation into the deoxyribonucleotide pool. In contrast, deoxyadenosine plus EHNA did not show this differential inhibition of [^3H]uridine incorporation into DNA, and the alteration in [^3H]cytidine incorporation into nucleotide pools was less impressive.

These data show an association between accumulation of dATP or dGTP and a primary inhibition of DNA synthesis, and they provide support for ribonucleotide reductase inhibition as the mechanism responsible for deoxyguanosine toxicity. Deoxyadenosine toxicity, however, appears to result from another, or perhaps a combination of, molecular event(s).

INTRODUCTION

The association of at least two inborn errors of purine metabolism with immunodeficiency diseases has made possible the investigation of these diseases at the molecular level. Adenosine deaminase (ADA)¹ deficiency results in severe combined immunodeficiency disease with impairment of T- and B-lymphocyte function (1). Purine nucleoside phosphorylase (PNP) deficiency has been associated with T-lymphocyte dysfunction in nine patients (2–9). The mechanism(s) whereby these enzyme deficiency states affect lymphocyte development and/or function has not been fully elucidated. However, the recent findings of markedly elevated dATP levels in ADA-deficient erythrocytes (10, 11) and of dGTP levels in PNP-deficient erythrocytes (12) in conjunction with high-circulating or urinary levels of the corresponding deoxyribonucleoside in these patients (3, 13) provide support for the hypothesis that deoxyribonucleosides play an important role in the

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¹Abbreviations used in this paper: ADA, adenosine deaminase; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; PCA, perchloric acid; PNP, purine nucleoside phosphorylase.

pathogenesis of the immune dysfunction in these two diseases and suggest that deoxyribonucleosides or their metabolites may be toxic to lymphoid cells.

The purine deoxyribonucleosides have indeed been shown to have lymphocytotoxic effects *in vitro*. Deoxyadenosine in the presence of an inhibitor of ADA inhibits [³H]thymidine incorporation into DNA by peripheral blood lymphocytes (14) and is toxic in micromolar concentrations to a variety of mouse (15, 16) and human (17, 18) lymphoid cell lines. Deoxyguanosine in the absence of a PNP inhibitor is likewise extremely toxic to lymphoid cells in culture (18–20). In addition, the rather striking selectivity of both deoxyadenosine and deoxyguanosine toxicity for human lymphoblasts of T-cell origin, as opposed to B-cell origin (18, 21), has provided a potential explanation for the prominent T-cell dysfunction in patients with ADA and PNP deficiency, respectively.

The cytotoxic effects of deoxyadenosine and deoxyguanosine on cultured cells correlate well with an accumulation of the corresponding deoxyribonucleoside triphosphate and are also associated with a decrease in the intracellular dCTP pool (15, 16, 19, 20). These observations, in conjunction with substantial reversal of toxicity in several culture systems with exogenous deoxycytidine (16–20), have led many investigators to suggest inhibition of the enzyme ribonucleotide reductase by dATP or dGTP as the mechanism underlying cellular toxicity (10, 16, 17, 20). Ribonucleotide reductase is responsible for catalyzing the reduction of all ribonucleoside diphosphates, and, thus, this enzyme appears to be important in the control of DNA synthesis. It is subject to strong negative allosteric control by deoxyribonucleoside triphosphates (22). Theoretically, therefore, accumulation of dATP or dGTP could inhibit ribonucleotide reductase, thus inhibiting the conversion of CDP to dCDP and dCTP. dCTP has been postulated to play a major regulatory role in the control of DNA synthesis in chinese hamster ovary cells, and a decrease in the dCTP pool correlates well with inhibition of DNA synthesis in these cells (23). Direct phosphorylation of exogenous deoxycytidine could bypass the requirement for ribonucleotide reductase, replenish the dCTP pool, and restore DNA synthesis. Despite the logic of this thesis, there is no direct evidence for inhibition of ribonucleotide reductase in intact human lymphoid cells under conditions that cause cellular toxicity, although such evidence does exist for deoxyguanosine-mediated toxicity in a mouse T-lymphoma line (24).

We have studied the effects of deoxyadenosine and deoxyguanosine on DNA synthesis in human T lymphoblasts to investigate more directly the molecular mechanism of deoxyribonucleoside toxicity in these cells. In addition, we have used the differential incorporation of several labeled ribonucleosides and

deoxyribonucleosides into DNA, into RNA, and into ribonucleotide pools to assess the effects of deoxyadenosine and deoxyguanosine on ribonucleotide reductase activity. Whereas our results provide more direct evidence for the inhibition of ribonucleotide reductase in deoxyguanosine toxicity, they also suggest that additional factors may play a role in the toxicity of deoxyadenosine.

METHODS

Radioisotopes and chemicals. [³H]Cytidine (28.0 Ci/mmol), [³H]uridine (24.2 Ci/mmol), [³H]deoxyuridine (21.9 Ci/mmol), [³H]dTTP (18.3 Ci/mmol), and [³H]dCTP (22.3 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.), and [U-¹⁴C]thymidine (515 mCi/mmol) and [U-¹⁴C]deoxycytidine (462 mCi/mmol) were obtained from Amersham Corp (Arlington Heights, Ill.). All unlabeled ribonucleosides and deoxyribonucleosides, *Micrococcus lysodeikticus* DNA polymerase, calf thymus DNA, and Dowex 50 × 4 200–400 (Dow Chemical Co., Midland, Mich.) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Affi-Gel 601 was obtained from Bio-Rad Laboratories (Richmond, Calif.). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was provided by Burroughs-Wellcome Co., Research Triangle Park, N. C.

Cell cultures. The Molt-4 T-lymphoblast line was obtained from Hem Research, Inc., Rockville, Md., and was originally derived from a patient with acute lymphoblastic leukemia. Cultures were maintained in exponential growth in RPMI-1640 medium plus 10% horse serum.

Deoxyribonucleotide pool measurements. Cells were incubated at a concentration of 10⁶/ml in RPMI plus 10% heat-inactivated horse serum at 37°C for 1 h with or without additives. Levels of dATP, dGTP, and dTTP were determined in 1 × 10⁶ cells; dCTP levels were determined in 1 × 10⁷ cells. At the end of the incubation period, cold RPMI medium (5 vol) was added. The cells were sedimented at 450 g for 5 min at 4°C and washed once with the same conditions in 1 ml 150 mM NaCl, 10 mM Tris, pH 7.4 (Buffer A). The intracellular deoxyribonucleotides were extracted from the cell pellet with 1 ml 60% methanol at –20°C for 12 h, the methanol evaporated, and the residue resuspended in 50 μl of distilled H₂O for assay. Deoxyribonucleoside triphosphate recovery under these conditions was 83 ± 5% (mean ± 1 SD; n = 5).

Deoxyribonucleoside triphosphate levels were determined with a modification of the DNA polymerase assay, as previously described (18). [³H]dTTP (3 Ci/mmol) was used for dATP, dGTP, and dCTP assays, and [³H]dCTP (3 Ci/mmol) for the dTTP assays. All assays were performed in duplicate with 0.3 U of DNA polymerase, 100 pmol of the nonlimiting deoxyribonucleotides, and 10 μl of cell extract in a final volume of 200 μl. Standard curves were linear from 2 to 10 pmol of limiting deoxyribonucleotide, and samples were diluted to fall within this range.

Radioisotope incorporation into DNA and RNA. After incubation of cells with labeled nucleosides, the RNA and DNA components of acid-precipitable cellular material were separated by methods similar to those described by Plagemann (25). Duplicate 1-ml aliquots of 1 × 10⁶ cells were harvested, washed with 1 ml of Buffer A, and precipitated with 1 ml of cold 0.5 N perchloric acid (PCA). All precipitates throughout the procedure were collected by centrifugation at 900 g for 6 min. The PCA precipitates were washed with 1 ml of cold 10% TCA and then with 1 ml of cold ether:ethanol (1:1). The RNA was hydrolyzed in 0.4 ml of 0.3 N KOH over 18 h at 37°C. The hydrolysate was cooled to 4°C, cold 10 N PCA (10 μl) was

added, and the mixture was centrifuged as above. The resulting supernate, which contained hydrolyzed RNA, was removed, immediately neutralized with 200 μ l of 0.7 N cold KOH, and recentrifuged to remove insoluble salts. Precipitated DNA from the KOH hydrolysis was washed twice with 2 ml of cold 10% TCA, resuspended in 0.6 ml of 10% TCA, and heated at 70°C for 45 min. The entire hydrolysate, which contained either DNA or RNA, was added to 10 ml of Aquasol (New England Nuclear) and counted in a Packard Tricarb scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Differential ^{14}C and ^3H incorporation into DNA was measured by the method of Kobayashi and Maudsley (26) with a counting efficiency of 21% for ^3H and 75% for ^{14}C . The sum of the radioactivities recovered in the RNA and DNA hydrolysates was $96 \pm 7\%$ (mean ± 1 SD; $n = 5$) of that recovered in the whole-cell TCA precipitate.

The distribution of [^3H]uridine incorporation into either the 2',3'-UMP or the 2',3'-CMP products of RNA hydrolysis was determined by the method of Katy and Comb (27); 2',3'-UMP was separated from 2',3'-CMP by cation exchange. The Dowex 50 column, 1 \times 5 cm (200–400 mesh, four times cross-linked), was washed with 20 ml of 3 N HCL, 20 ml of H_2O , and 20 ml of 0.05 N HCL between samples. The flow rate was 0.5 ml/min, and 1-ml fractions were collected.

The RNA hydrolysate (0.6 ml of 0.3 N KOH) of cells labeled with [^3H]uridine (obtained as described above) was acidified at 0°C with 10 N PCA (37 μ l) and centrifuged at 900 g for 5 min at 4°C. The supernate was neutralized to pH 5–8 with 1 N KOH at 0°C, centrifuged, and 200 μ l of the supernate was diluted with 200 μ l of 0.1 N HCL before application on the column. The 2',3'-UMP component was collected by washing with 6 ml of 0.05 N HCL; the 2',3'-CMP component was eluted with 10 ml of 0.5 N NaOH. The 1-ml fractions were counted directly in 10 ml Aquasol. Recoveries of labeled UMP and CMP ranged from 90 to 100%.

Ribonucleotide and deoxyribonucleotide separations. An affinity gel (Affi-Gel 601) that selectively binds cis-diol compounds at high pH was used to separate deoxyribonucleotides from ribonucleotides (Fig. 1). The gel was suspended in 50 mM Hepes, pH 8.5, and 50 mM MgCl_2 (Buffer B) and packed in a 1-cm diameter column at a total volume of 3 ml. Flow rate was 0.5 ml/min, and 1-ml fractions were collected.

Molt-4 cells labeled with [^3H]cytidine were washed with 2 ml of Buffer A and extracted in 60% methanol as described above, the methanol was evaporated, and the residue was resuspended in 0.5 ml of Buffer B and applied to the column. Deoxyribonucleotides were collected by washing with 10 ml of Buffer B; the ribonucleotides were subsequently eluted from the column with 50 mM of sodium phosphate buffer, pH 6.0. Similar results were obtained when 100 mM of sodium acetate, pH 5.0, was used as the eluting buffer. The 1-ml fractions were counted directly in 10 ml of Aquasol. Recoveries of labeled ribo- and deoxyribonucleotides were consistently in the range of 95–100%.

RESULTS

Effects of deoxyadenosine and deoxyguanosine on deoxyribonucleotide pools, DNA synthesis, and RNA synthesis. Incubation of cells with 5 μM EHNA plus 50 μM deoxyadenosine inhibited the incorporation of [^3H]thymidine, [^3H]uridine, [^3H]deoxyuridine, [^3H]cytidine, and [^{14}C]deoxycytidine into DNA (Table I). Control experiments showed little effect of 5 μM EHNA or of 50 μM deoxyadenosine alone on DNA synthesis. Deoxyguanosine alone (50

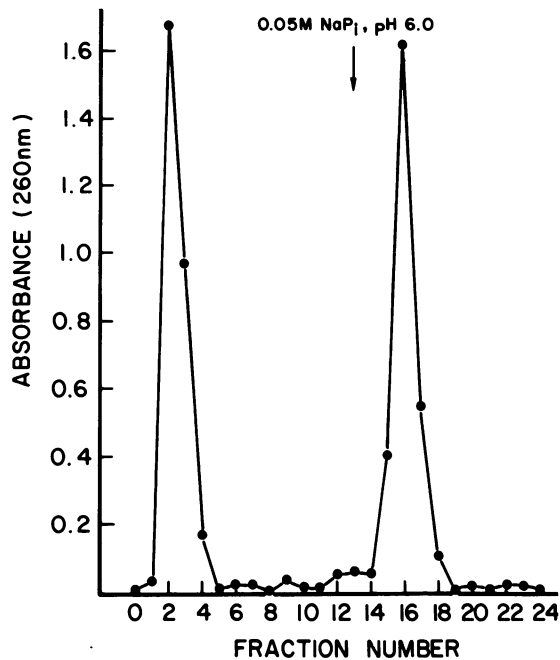


FIGURE 1 Chromatographic separation of ribonucleotides and deoxyribonucleotides. A standard that contained 0.33 μmol each of the ribonucleotides, CMP, CDP, and CTP, and/or the deoxyribonucleotides, dCMP, dCDP, and dCTP, was applied in 200 μl of Buffer A to a 1 \times 4-cm column of Affi-gel 601. The absorbance of each 1-ml fraction was monitored at 260 nm. Deoxyribonucleotides were consistently recovered in the void volume (fractions 1–4). Ribonucleotides were eluted with 0.05 M sodium phosphate (NaPi), pH 6.0 (fractions 14–18).

μM) also inhibited the incorporation of [^3H]thymidine, [^3H]deoxyuridine, and [^3H]uridine into DNA.

As shown in Table II, incubation of Molt-4 lymphoblasts with increasing concentrations of deoxyadenosine from 0 to 50 μM in the presence of 5 μM EHNA for 1 h resulted in a fivefold increment in dATP levels and a concomitant depletion of the dCTP pool to undetectable levels. dTTP and dGTP pools remained relatively constant. [^3H]Uridine was added to these cells for an additional 2.5-h incubation period. There was a reduction in the incorporation of this isotope into DNA with increasing concentrations of deoxyadenosine, but incorporation into RNA was not decreased.

Similar experiments were carried out with deoxyguanosine (0–50 μM) alone (Table III). As deoxyguanosine concentrations were increased from 5 to 25 μM , intracellular levels of dGTP rose more than fivefold and there was a substantial fall in the incorporation of [^3H]uridine into DNA. There was no decline in the incorporation of [^3H]uridine into RNA. dCTP levels fell as the concentration of deoxyguanosine was increased, whereas dTTP levels remained relatively

TABLE I
Effects of Deoxyadenosine and Deoxyguanosine on Incorporation of Nucleotides into DNA

Additive	Nucleoside incorporation into DNA				
	[³ H]dThd	[¹⁴ C]dCyd	[³ H]Cyd	[³ H]dUrd	[³ H]Urd
	% control				
EHNA (5 μM)	102±6 (4)	97±4 (4)	102, 97 (2)	100, 99 (2)	—
Deoxyadenosine (50 μM)	94±10 (8)	75±2 (6)	114, 96 (2)	86±4 (4)	85±6 (4)
EHNA (5 μM) + deoxyadenosine (50 μM)	9±10 (8)	23±7 (4)	24, 21 (2)	6±1 (4)	10±3 (4)
Deoxyguanosine (50 μM)	25±1 (4)	—	—	28, 27 (2)	10, 2, 6 (3)

Cells were incubated at a concentration of 1×10^6 /ml in RPMI plus 10% heat-inactivated horse serum for 1 h in the presence of the additive indicated. Labeled nucleosides were added for a further 75-min incubation period at concentrations of 0.1 μM. Nucleoside specific activities: [³H]dThd (9 Ci/mmol), [¹⁴C]dCyd (185 mCi/mmol), [³H]Cyd (5.6 Ci/mmol), [³H]dUrd (4.4 Ci/mmol), and [³H]Urd (8.1 Ci/mmol). Values for four or more determinations represent the mean ± 1 SD. Mean control counts for experiments: [³H]dThd (19,468 cpm), [¹⁴C]dCyd (7,310 cpm), [³H]Cyd (2,864 cpm), [³H]dUrd (13,597 cpm), and [³H]Urd (8,274 cpm).

constant. dATP levels, on the other hand, increased progressively from 66 to 110 pmol/10⁶ cells.

The relative incorporation of [³H]uridine into either the cytosine or uracil residues of RNA was determined by analysis of the distribution of incorporation of [³H]-uridine into the 2',3'-UMP and 2',3'-CMP components of the RNA hydrolysate (Table IV). T lymphoblasts incubated with [³H]uridine for 75 and 150 min incorporated 75 and 66% of the total radioactivity into the 2',3'-UMP component of the hydrolysate. Preincubation with deoxyadenosine (50 μM) plus EHNA (5 μM) increased the total incorporation of [³H]uridine into RNA; most of the radioactivity was still in the 2',3'-UMP

component of the RNA hydrolysate after either a 75- (86%) or a 150-min (77%) incubation with [³H]uridine. Similar results were obtained when cells were preincubated with deoxyguanosine (50 μM).

Differential effects of hydroxyurea, deoxyguanosine, and deoxyadenosine plus EHNA on ribonucleoside and deoxyribonucleoside incorporation into DNA. Simultaneous measurements of the incorporation of [³H]-deoxyuridine and [¹⁴C]thymidine into DNA were compared with the incorporation of [³H]uridine and [¹⁴C]thymidine into DNA in the presence of increasing concentrations of hydroxyurea, deoxyguanosine, or deoxyadenosine plus 5 μM EHNA. Hydroxyurea at con-

TABLE II
Effects of Increasing Deoxyadenosine Concentrations on Deoxyribonucleotide Pools, DNA Synthesis, and RNA Synthesis

Deoxyadenosine concentration μM	dATP	dGTP	dCTP	dTTP	[³ H]Uridine into DNA		[³ H]Uridine into RNA	
	pmol/10 ⁶ cells				cpm	% control	cpm	% control
0	66±5*	24±3*	2.5±0.3*	37±9‡	27,822	100	95,430	100
1	57	23	2.1	36	27,491	99	100,686	105
5	70	9	2.0	39	23,483	84	102,575	107
10	109	13	1.5	34	17,473	63	114,184	119
25	206	15	0.1	44	4,509	16	109,730	115
50	318	15	<0.1	46	2,226	8	105,927	111

Cells were incubated at a concentration of 10⁶/ml in RPMI plus 10% heat-inactivated horse serum for 1 h with 5 μM of EHNA and deoxyadenosine at the indicated concentrations. [³H]Uridine (40 nM, 20 Ci/mmol) was added for an additional 2.5 h, and incorporation into RNA and DNA was measured as detailed in Methods.

* Mean ± SD in four experiments.

‡ Mean ± SD in 12 experiments.

TABLE III

Effects of Increasing Deoxyguanosine Concentrations on Deoxyribonucleotide Pools, DNA Synthesis, and RNA Synthesis

Deoxyadenosine concentration μM	dATP	dGTP	dCTP	dTTP	^3H Uridine into DNA		^3H Uridine into RNA	
					cpm	% control	cpm	% control
		pmol/10 ⁶ cells						
0	66±5*	24±3*	2.5±0.3*	37±9‡	20,601	100	74,269	100
1	—	—	—	—	20,921	98	79,659	107
5	79	31	1.3	44	15,201	74	88,241	120
10	91	79	1.2	39	4,663	22	89,241	120
25	102	174	1.1	40	1,568	8	89,494	120
50	110	305	0.3	31	443	2	82,814	112

Cells were incubated as detailed in Table I with increasing concentrations of deoxyguanosine rather than deoxyadenosine and EHNA.

* Mean±SD in four experiments.

‡ Mean±SD in 12 experiments.

centrations from 100 μM to 5 mM progressively inhibited incorporation of both ^3H deoxyuridine and ^{14}C thymidine into DNA (Fig. 2A). Incorporation of ^3H uridine, however, had fallen to 20% of control values at 100 μM hydroxyurea, a concentration that left ^{14}C thymidine incorporation relatively unaffected (Fig. 2B). Deoxyguanosine alone exhibited effects similar qualitatively to those observed with hydroxyurea. Incorporation of ^3H uridine into DNA was markedly inhibited at a concentration of 10 μM , whereas incorporation of thymidine and deoxyuridine into DNA was inhibited to a similar degree only at deoxyguanosine concentrations of 50 μM or above (Fig. 3). Similar results were obtained with deoxyguanosine in the presence of EHNA (data not shown). In contrast to these findings with hydroxyurea or deoxyguanosine, deoxyadenosine plus 5 μM EHNA inhibited the incorporation of labeled uridine into DNA to an extent similar to that observed with either

^{14}C thymidine or ^3H deoxyuridine (Fig. 4). In addition, there was no differential inhibition of incorporation of ^3H cytidine as compared with ^{14}C deoxycytidine into DNA under these conditions (data not shown). To control for potential artifacts induced by the double-labeling technique, the simultaneous incorporations of ^3H thymidine and of ^{14}C thymidine were measured in the presence of increasing concentrations of hydroxyurea and were found to be identical (data not shown).

Effects of hydroxyurea, deoxyguanosine, and deoxyadenosine plus EHNA on incorporation of ^3H -cytidine into ribonucleotide pools. Cells were preincubated for 1 h with deoxyribonucleoside (50 μM) or hydroxyurea (2.5 mM) at concentrations known to inhibit ^3H uridine incorporation into DNA by >90%. Intracellular ribo- or deoxyribonucleotides were extracted in 60% methanol and separated by affinity chromatography as described in Methods.

TABLE IV
 ^3H Uridine Incorporation into the UMP and CMP Components of RNA

Additives	Time	^3H Uridine into 2',3'-UMP		^3H Uridine into 2',3'-CMP	
		min	cmp	% total	cmp
None	75	22,252	75	7,272	25
	150	34,817	66	18,041	34
Deoxyadenosine (50 μM) + EHNA (5 μM)	75	27,339	86	4,363	14
	150	50,712	77	14,816	23
Deoxyguanosine (50 μM)	75	31,425	84	5,987	16
	150	52,539	77	15,269	23

Cells were incubated at a concentration of $1 \times 10^6/\text{ml}$ in RPMI plus 10% heat-inactivated horse serum for 1 h in the presence of the additive indicated. ^3H Uridine (40 nM, 20 Ci/mmol) was added to 3-ml cultures for either 75 or 150 min. The incorporation of ^3H uridine into the 2',3'-UMP and the 2',3'-CMP components of the RNA hydrolysate was determined as described in Methods.

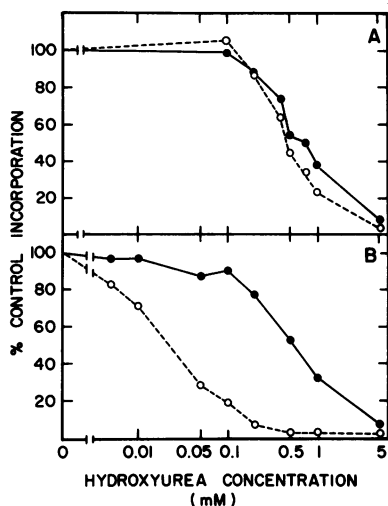


FIGURE 2 Differential incorporation of nucleosides into DNA: effects of increasing concentrations of hydroxyurea. Cells were incubated at a concentration of 1×10^6 /ml in RPMI plus 10% heat-inactivated horse serum in the presence of the hydroxyurea concentration indicated for 1 h. Labeled nucleosides were added for an additional 2.5-h incubation period. (A) ●—●, [^{14}C]thymidine (50 nM, 515 mCi/mmol); ○---○, [^3H]deoxyuridine (50 nM, 9 Ci/mmol). (B) ●—●, [^{14}C]thymidine, as in Panel A; ○---○, [^3H]uridine (40 nM, 24.2 Ci/mmol). Values are expressed as a percentage of control values obtained in the absence of additives, and are the mean of duplicate determinations of a single experiment. Similar results were obtained in two additional experiments. The mean control cpm \pm 1 SD for 12 determinations: (A) [^{14}C]thymidine $7,442 \pm 321$, [^3H]deoxyuridine $9,072 \pm 389$; (B) [^{14}C]thymidine $7,442 \pm 492$, [^3H]uridine $11,284 \pm 551$.

After incubation with hydroxyurea, there was a decrease in the incorporation of [^3H]cytidine into deoxyribonucleotides to a mean of 31% of control values and a corresponding increase to 144% of control in the labeled ribonucleotide pool (Table V); almost identical results were obtained with deoxyguanosine. Incubation with deoxyadenosine plus EHNA, however, yielded intermediate results with a less pronounced fall in counts in the deoxyribonucleotide fraction (70% of control) and a more modest increment in the ribonucleotide component (110% of control).

DISCUSSION

Deoxyribonucleoside toxicity for lymphoid cells is currently felt to account, at least in part, for the immunodeficiency syndromes associated with ADA and PNP deficiency. We have previously demonstrated marked cytotoxicity of deoxyadenosine plus EHNA and of deoxyguanosine alone for human T lymphoblasts, an effect which is significantly greater than the toxicity of these compounds for cultured human B lymphoblasts. In this study, we have focused on the mechanism of toxicity of deoxyribonucleosides in human T lympho-

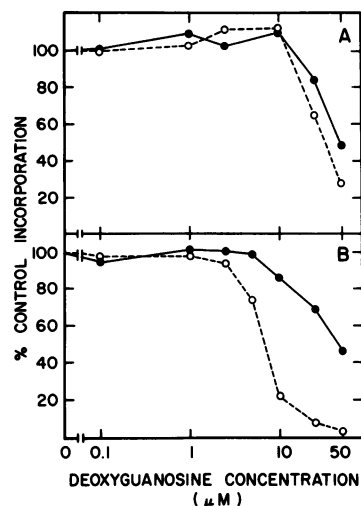


FIGURE 3 Differential incorporation of nucleosides into DNA: effects of increasing concentrations of deoxyguanosine. Experimental conditions and symbols are identical to those in Fig. 2.

blasts because it is this effect that appears to be of major clinical importance.

We have examined the effects of deoxyribonucleosides on the simultaneous incorporation of [^3H]uridine into RNA and into DNA in cultured T cells. Because the incorporation of any nucleoside is a function not only of nucleic acid synthesis, but also of nucleoside transport, phosphorylation, and dilution in an unlabeled nucleotide pool, incorporation studies in the presence of exogenous deoxyribonucleosides that may interfere with these processes must be stringently

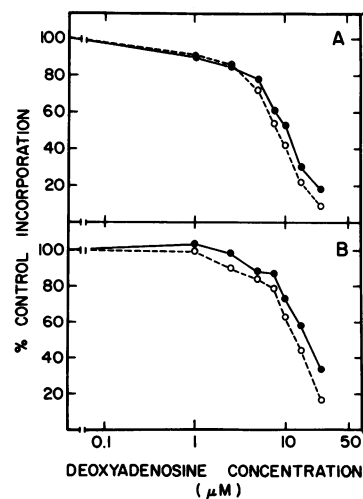


FIGURE 4 Differential incorporation of nucleosides into DNA: effects of increasing concentrations of deoxyadenosine. Experimental conditions and symbols are identical to those in Fig. 2.

TABLE V
³H]Cytidine Incorporation into Ribonucleotide and Deoxyribonucleotide Pools in T Lymphoblasts

Additives	Experiment (n)	Deoxyribonucleotides	Ribonucleotides
		% control	% control
None	1 (3)	100	100
	2 (2)	100	100
	3 (3)	100	100
Hydroxyurea (2.5 mM)	1 (2)	25	150
	2 (1)	26	142
	3 (2)	42	139
Deoxyguanosine (50 μM)	1	—	—
	2 (1)	33	152
	3 (2)	41	137
Deoxyadenosine (50 μM) + EHNA (5 μM)	1 (2)	60	109
	2	—	—
	3 (2)	79	110

Molt-4 lymphoblasts (10⁶ cells/ml) were incubated in 2.5 ml of RPM1 plus 10% heat-inactivated horse serum for 1 h at 37°C in the presence of the additives indicated. ³H]Cytidine (28 Ci/mmol; 50 nM in experiments 1 and 2, 0.12 μM in experiment 3) was added for a further 75-min incubation period. *n* = number of determinations per experiment. Control values: experiment 1, deoxyribonucleotides 3,175 cpm, ribonucleotides 46,419 cpm; experiment 2, deoxyribonucleotides, 2,761 cpm, ribonucleotides 88,005 cpm; experiment 3, deoxyribonucleotides 9,753 cpm, ribonucleotides 212,050 cpm.

controlled. Comparison of the incorporation of ³H]uridine into DNA and RNA allows us to control for interference with both the transport and the phosphorylation of this labeled nucleoside. Our results support a progressive inhibition of DNA synthesis, but not of RNA synthesis, by increasing concentrations of deoxyadenosine plus EHNA and deoxyguanosine. Our studies confirm and expand those reported by others in which the toxic effect of deoxyadenosine plus EHNA on mouse leukemia cells was evaluated with microfluorometry, as well as the incorporation of ³H]thymidine into DNA and ³H]uridine into RNA (15).

One hypothesis to account for deoxyribonucleoside-mediated inhibition of DNA synthesis is an inhibition of the enzyme ribonucleotide reductase by elevated levels of dATP or dGTP. This enzyme catalyzes the reduction of ribonucleoside diphosphates to their corresponding 2'-deoxy derivatives and insures an adequate supply of deoxyribonucleotides for DNA synthesis. In partially purified cell extracts of Novikoff rat tumor cells, dATP has been demonstrated to inhibit all reductions catalyzed by this enzyme, whereas dGTP inhibits the reduction of CDP, GDP, and UDP, but may stimulate the reduction of ADP (22). In our study, we have documented a progressive increase in dATP or dGTP levels in T lymphoblasts and a decline in the much smaller dCTP pool in the presence of an in-

creasing concentration of deoxyadenosine or deoxyguanosine, respectively. These results confirm those reported for other cell systems at isolated high concentrations of deoxyribonucleosides (15, 16, 19, 20). In contrast to other studies, however, we have observed no definite effect of either deoxyribonucleoside tested on dTTP pools and, in the presence of deoxyguanosine, we have noted a twofold increase in the dATP pool, an effect consistent with the putative activation of ribonucleotide reductase by dGTP.

The depletion of dCTP and the reversal of toxicity with deoxycytidine, i.e., deoxycytidine rescue, have frequently been cited as the major evidence for ribonucleotide reductase inhibition in deoxyribonucleoside toxicity (16, 17, 20). Such an interpretation may be erroneous, because the reversal or prevention of deoxyribonucleoside-mediated toxicity by deoxycytidine could be attributed to a number of mechanisms other than bypass of the block created by inhibition of ribonucleotide reductase. For example, the effect of deoxycytidine could be a result of inhibition of deoxyadenosine or deoxyguanosine phosphorylation. There is good evidence for the identity of deoxycytidine kinase and deoxyguanosine kinase in mouse T-lymphoma cells (20), and deoxycytidine could completely prevent toxicity by competitive inhibition of deoxyguanosine phosphorylation. Whereas deoxy-

adenosine kinase activity appears to reside in two separate enzymes in this system (16) and may not be totally inhibited by deoxycytidine, dCTP can inhibit deoxyadenosine phosphorylation in cell extracts (28); thus, an increase in the intracellular dCTP pool after the addition of deoxycytidine could prevent an accumulation of dATP.

Because deoxycytidine rescue could be a result of a number of different mechanisms and thus is poor evidence for ribonucleotide reductase inhibition and because dCTP depletion could also result from several of the alternatives suggested, we have tested more directly the hypothesis that deoxyribonucleoside toxicity is mediated by an inhibition of this enzyme. In addition, we have compared the effects of deoxyguanosine with the effects of hydroxyurea, a known inhibitor of ribonucleotide reductase (29–31), on the incorporation of ribonucleosides and deoxyribonucleosides into DNA.

Ribonucleotide reductase activity is required for the incorporation of uridine, but not of deoxyuridine or thymidine, into DNA (Fig. 5). Inhibition of this enzyme would thus be expected to result in a greater decrease in the incorporation of uridine than of either deoxyuridine or thymidine into DNA. Just such a differential effect was observed with hydroxyurea at concentrations in the millimolar range and with deoxyguanosine at concentrations in the micromolar range. Although these

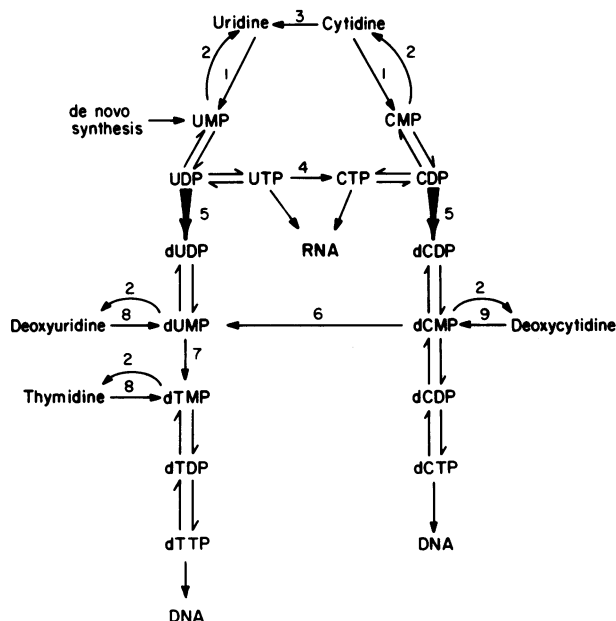


FIGURE 5 Pathways of cytidine, uridine, deoxyuridine, and thymidine incorporation into nucleic acids. 1, uridine kinase; 2, 5'-nucleotidase; 3, cytidine deaminase; 4, CTP synthetase; 5, ribonucleotide reductase; 6, dCMP deaminase; 7, thymidylate synthetase; 8, thymidine kinase.

data are consistent with an inhibition of ribonucleotide reductase, one must consider other possibilities. Very little [^3H]uridine is incorporated into the cytosine residues of RNA (Table IV), which suggests that only a small fraction of [^3H]UTP is converted via CTP synthetase (reaction 4, Fig. 5) into the cytidine nucleotide pools. Thus, it is highly unlikely that a significant amount of [^3H]uridine is incorporated into DNA via dCTP under the conditions of our double-label experiments. Assuming that the majority of [^3H]uridine is incorporated into DNA via dTTP, one may conclude that the site of the differential inhibition of [^3H]uridine incorporation as compared with [^{14}C]thymidine incorporation into DNA must occur between transport of uridine and the phosphorylation of dTMP (Fig. 5). Because uridine incorporation into RNA is not inhibited, uridine phosphorylation and transport, and UMP phosphorylation are unimpaired. We have excluded involvement of thymidylate synthetase (reaction 7, Fig. 5) by demonstrating no difference in the inhibition of incorporation of deoxyuridine as compared with thymidine into DNA in cells incubated with either deoxyguanosine or hydroxyurea. Our data might also be explained by a relative stimulation of thymidine and deoxyuridine incorporation into DNA as a result of decreased feedback inhibition of thymidine kinase (reaction 8, Fig. 5) by depleted dTTP pools (32). However, we have found that dTTP pools remain relatively constant with increasing dGTP levels.

After careful consideration of these alternative explanations, we conclude that our results are due to inhibition of either the reduction of UDP by ribonucleotide reductase or of the reversible conversion of dUDP to dUMP catalyzed by thymidylate kinase. Because thymidylate kinase does not appear to be regulated allosterically by nucleotides (33), it is most probable that our results are explained by an inhibition of ribonucleotide reductase.

Further support for this conclusion can be derived from our studies on the incorporation of [^3H]cytidine into ribonucleotide and deoxyribonucleotide pools. [^3H]Cytidine must be phosphorylated to CDP and reduced by ribonucleotide reductase to enter the deoxyribonucleotide pool (Fig. 5). A block of ribonucleotide reductase should substantially increase counts appearing in the ribonucleotide fractions while decreasing counts in the deoxyribonucleotide pool. This result was obtained with both hydroxyurea and deoxyguanosine.

Deoxyadenosine plus EHNA, in contrast to either hydroxyurea or deoxyguanosine, produced little, if any, differential effect on the incorporation of uridine and thymidine into DNA. In addition, there was a far more modest increment in the ribonucleotide pool at a concentration of deoxyadenosine which effectively inhibited DNA synthesis.

Interpretation of these results requires careful consideration of possible changes in the dTTP pool. An increasing pool of dTTP could inhibit the incorporation of thymidine and deoxyuridine into DNA as a result of greater feedback inhibition of thymidine kinase and/or dilution of the labeled dTTP formed. This seems unlikely because we found no significant elevation of the dTTP pool after a 1-h incubation with 50 μ M deoxyadenosine plus 5 μ M EHNA. In addition, we controlled for possible changes in pool size by comparing both [3 H]uridine and [3 H]deoxyuridine incorporation with [14 C]thymidine incorporation into DNA.

Because [3 H]uridine can be incorporated into DNA through both the dTTP and dCTP pools (Fig. 5), it is also conceivable that deoxyadenosine and deoxyguanosine could have different effects on the relative distribution of [3 H]uridine into these nucleotide pools. This might, in turn, account for variable dilution of the tritium label in pools of different sizes and an alteration in the incorporation of tritium into DNA. We have found that the incorporation of [3 H]uridine into the cytidine nucleotide pool in the presence of deoxyadenosine is small, and is identical to that obtained with deoxyguanosine. These experiments demonstrate that the major route of [3 H]uridine incorporation into DNA is through the dTTP pool, which remains constant in size in the presence of both deoxyadenosine and deoxyguanosine. In view of these controls, our data suggest that deoxyadenosine, in the presence of an inhibitor of adenosine deaminase, may inhibit DNA synthesis by mechanisms other than, or, in addition to, inhibition of ribonucleotide reductase. One recently espoused possibility is that an increased concentration of deoxyadenosine leads to an accumulation of S-adenosylhomocysteine by suicide inactivation of S-adenosylhomocysteine hydrolase, thereby depleting the cell of S-adenosylmethionine and preventing essential methylation reactions (34).

The apparent difference in the mechanisms of toxicity of deoxyguanosine and deoxyadenosine could account for the clinical differences in the syndromes associated with PNP deficiency (isolated T-cell dysfunction) and ADA deficiency (combined T- and B-cell dysfunction).

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REFERENCES

1. Giblett, E. R., J. E. Anderson, F. Cohen, B. Pollara, and H. J. Meuwissen. 1972. Adenosine deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet*. **II**: 1067–1069.
2. Giblett, E. R., A. J. Ammann, D. W. Wara, R. Sandman,

- and L. K. Diamond. 1975. Nucleoside phosphorylase deficiency in a child with severely defective T-cell immunity and normal B-cell immunity. *Lancet*. **I**: 1010–1013.
3. Cohen, A., D. Doyle, D. W. Martin, Jr., and A. J. Ammann. 1976. Abnormal purine metabolism and purine overproduction in a patient deficient in purine nucleoside phosphorylase. *N. Engl. J. Med.* **295**: 1449–1454.
4. Stoop, J. W., V. P. Eysvoogel, B. J. M. Zegers, B. Blokschut, D. W. van Bekkum, and R. E. Ballieux. 1976. Selective severe cellular immunodeficiency: effect of thymus transplantation and transfer factor administration. *Clin. Immunol. Immunopathol.* **6**: 289–298.
5. Stoop, J. W., B. J. M. Zegers, G. F. M. Hendrickx, L. H. Siegenbeek van Heukelom, G. E. J. Staal, P. K. deBree, S. K. Wadman, and R. E. Ballieux. 1977. Purine nucleoside phosphorylase deficiency associated with selective cellular immunodeficiency. *N. Engl. J. Med.* **296**: 651–655.
6. Edwards, N. L., E. W. Gelfand, D. Biggar, and I. H. Fox. 1978. Partial deficiency of purine nucleoside phosphorylase: studies of purine and pyrimidine metabolism. *J. Lab. Clin. Med.* **91**: 736–749.
7. Virelizier, J. L., M. Hamet, J. J. Ballet, P. Reinert, and C. Griscelli. 1978. Impaired defense against vaccinia in a child with T-lymphocyte deficiency associated with inosine phosphorylase defect. *J. Pediatr.* **92**: 358–362.
8. Carapella-de Luca, E., F. Aiuti, P. Lucarelli, L. Bruni, C. D. Baroni, C. Imperato, D. Roos, and A. Astaldi. 1978. A patient with nucleoside phosphorylase deficiency, T-cell deficiency, and hemolytic anemia. *J. Pediatr.* **93**: 1000–1003.
9. Rich, K. C., W. J. Arnold, T. Palella, and I. H. Fox. 1979. Cellular immune deficiency with autoimmune hemolytic anemia in purine nucleoside phosphorylase deficiency. *Am. J. Med.* **67**: 172–176.
10. Coleman, M. S., J. Donofrio, J. J. Hutton, L. Hahn, A. Daoud, B. Lampkin, and J. Dyminski. 1978. Identification and quantitation of adenine deoxynucleotides in erythrocytes of a patient with adenosine deaminase deficiency and severe combined immunodeficiency. *J. Biol. Chem.* **253**: 1619–1626.
11. Cohen, A., R. Hirschhorn, S. D. Horowitz, A. Rubinstein, S. H. Polmar, R. Hong, and D. W. Martin, Jr. 1978. Deoxyadenosine triphosphate as a potentially toxic metabolite in adenosine deaminase deficiency. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 472–476.
12. Cohen, A., L. J. Gudas, A. J. Ammann, G. E. J. Staal, and D. W. Martin, Jr. 1978. Deoxyguanosine triphosphate as a possible toxic metabolite in the immunodeficiency associated with purine nucleoside phosphorylase deficiency. *J. Clin. Invest.* **61**: 1405–1409.
13. Donofrio, J., M. S. Coleman, J. J. Hutton, A. Daoud, B. Lampkin, and J. Dyminski. 1978. Overproduction of adenine deoxynucleosides and deoxynucleotides in adenosine deaminase deficiency with severe combined immunodeficiency disease. *J. Clin. Invest.* **62**: 884–887.
14. Simmonds, H. A., G. S. Panayi, and V. Corrigan, 1978. A role for purine metabolism in the immune response: adenosine deaminase activity and deoxyadenosine catabolism. *Lancet*. **I**: 60–63.
15. Lowe, J. K., B. Gowans, and L. Brox. 1977. Deoxyadenosine metabolism and toxicity in cultured L5178Y cells. *Cancer Res.* **37**: 3013–3017.
16. Ullman, B., L. J. Gudas, A. Cohen, and D. W. Martin, Jr. 1978. Deoxyadenosine metabolism and cytotoxicity in cultured mouse T lymphoma cells: a model for immunodeficiency disease. *Cell*. **14**: 365–375.

17. Carson, D. A., J. Kaye, and J. E. Seegmiller. 1977. Lymphospecific toxicity in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency: possible role of nucleoside kinase(s). *Proc. Natl. Acad. Sci. U. S. A.* **74**: 5677-5681.
18. Mitchell, B. S., E. Mejias, P. E. Daddona, and W. N. Kelley. 1978. Purinogenic immunodeficiency diseases: selective toxicity of deoxyribonucleosides for T cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 5011-5014.
19. Chan, T. 1978. Deoxyguanosine toxicity on lymphoid cells as a cause for immunosuppression in purine nucleoside phosphorylase deficiency. *Cell*. **14**: 523-530.
20. Gudas, L. J., B. Ullman, A. Cohen, and D. W. Martin, Jr. 1978. Deoxyguanosine toxicity in a mouse T lymphoma: relationship to purine nucleoside phosphorylase-associated immune dysfunction. *Cell*. **14**: 531-538.
21. Carson, D. A., J. Kaye, and J. E. Seegmiller. 1978. Differential sensitivity of human leukemic T cell lines and B cell lines to growth inhibition by deoxyadenosine. *J. Immunol.* **121**: 1726-1731.
22. Moore, E. C., and R. B. Hurlbert. 1966. Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. *J. Biol. Chem.* **241**: 4802-4809.
23. Bjursell, G., and P. Reichard. 1973. Effects of thymidine on deoxyribonucleoside triphosphate pools and deoxyribonucleic acid synthesis in chinese hamster ovary cells. *J. Biol. Chem.* **248**: 3904-3909.
24. Ullman, B., L. G. Gudas, S. M. Clift, and D. W. Martin, Jr. 1979. Isolation and characterization of purine nucleoside phosphorylase-deficient T-lymphoma cells and secondary mutants with altered ribonucleotide reductase: genetic model for immunodeficiency disease. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 1074-1078.
25. Plagemann, P. G. W. 1971. Nucleotide pools of Novikoff rat hepatoma cells growing in suspension culture. *J. Cell. Physiol.* **77**: 213-240.
26. Kobayashi, Y., and D. V. Maudsley. 1970. Practical aspects of double-isotope counting. In *Current Aspects of Liquid Scintillation Counting*. E. Bransome, Jr., editor. Grune & Stratton, New York. 76-85.
27. Katy, K., and D. G. Comb. 1963. A new method for the determination of the base composition of ribonucleic acid. *J. Biol. Chem.* **238**: 3065-3067.
28. Krygier, V., and R. L. Momparler. 1971. Mammalian deoxynucleoside kinases. *J. Biol. Chem.* **246**: 2752-2757.
29. Adams, R. L. P., and J. G. Lindsay. 1967. Hydroxyurea: reversal of inhibition and use as a cell-synchronizing agent. *J. Biol. Chem.* **242**: 1314-1317.
30. Miller, M. R., J. J. Castellot, Jr., and A. B. Pardee. 1978. A permeable animal cell preparation for studying macromolecular synthesis, DNA synthesis and the role of deoxyribonucleotides in S phase initiation. *Biochemistry*. **17**: 1073-1080.
31. Lewis, W. H., and J. A. Wright. 1979. Isolation of hydroxyurea-resistant CHO cells with altered levels of ribonucleotide reductase. *Somatic Cell Genet.* **5**: 83-96.
32. Bresnick, E., and U. B. Thompson. 1965. Properties of deoxythymidine kinase partially purified from animal tumors. *J. Biol. Chem.* **240**: 3967-3974.
33. Henderson, J. F., and A. R. P. Paterson, 1973. *Nucleotide Metabolism: An Introduction*. Academic Press, Inc., New York. 238-241.
34. Hershfield, M. S. 1979. Apparent suicide inactivation of human lymphoblast S-adenosylhomocysteine hydrolase by 2'-deoxyadenosine and adenine arabinoside: a basis for direct toxic effects of analogs of adenosine. *J. Biol. Chem.* **254**: 22-25.