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Metabolism and Selectivity of Arabinonucleoside in Human Lymphoid Cells[1] (42123)

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Abstract. The selective toxicity of purine deoxynucleosides against lymphoid cells appears to be mediated by a preferential accumulation of the corresponding triphosphates in these cells. We report a study of the metabolism and toxicity of arabinonucleosides of guanine and cytosine toward human T- and B-lymphoblastoid-cell lines. Both compounds inhibited the growth of T lymphoblasts at concentrations < 2 μM. However, only ara-G exhibited a strong selectivity for T lymphocytes as indicated by a 100-fold greater toxicity to T than B cells. ara-G is not significantly degraded to guanine but is metabolized to the triphosphate. In common with the other arabinonucleoside, cytotoxicity by ara-G was associated with specific inhibition of DNA synthesis in cells. The capacity of T cells (CCRF-CEM) to accumulate ara-GTP was dependent primarily on deoxycytidine kinase. The level of intracellular ara-GTP accumulated after incubation with the corresponding nucleoside was 20- to 40-fold higher in T cells than either of two B-lymphoblast-cell lines, WI-L2 or PF-2S. The levels of phosphorylating activity for ara-C in extracts of T- and B-cell lines were approximately equal; in contrast, ara-G phosphorylating activity was four- to fivefold higher in B lymphoblasts. After removal of arabinonucleosides from the culture medium, ara-GTP levels in B lymphoblasts declined at a rate that was two to four times faster than that of ara-CTP. In marked contrast, no catabolism of the arabinonucleoside triphosphates was detected in T lymphoblasts. These results suggest that the selectivity of arabinonucleosides to human lymphoid cells of various phenotypes can be correlated with their nucleotide metabolism. The selectivity of ara-G for T and B cells can be correlated with their differential ability to catabolize ara-GTP. © 1985 Society for Experimental Biology and Medicine.

Deoxyribonucleosides can selectively impair lymphocyte growth and function. Inherited abnormalities in dAdo and dGuo metabolism have been associated with impaired immune function and selective destruction of T lymphocytes (1, 2). In these diseases lymphotoxicity appears to be mediated by an excessive accumulation of deoxyadenosine triphosphates or deoxyguanosine triphosphates in target tissues (3–5).

The finding that lymphoid tissues are sensitive to deoxynucleosides has encouraged the search for analogs that might mimic the effect of the natural nucleosides and selectively inhibit the growth of malignant T lymphocytes. It has been reported that the arabinonucleoside of guanine (ara-G)[2] preferentially inhibited the growth of malignant human T lymphoblastoid cells (6). These results suggested the idea that ara-G might have the necessary selectivity for neoplastic cells to be clinically useful as an anticancer or immunosuppressive agent (6). We have studied the metabolism and toxicity of ara-G on multiple established lines of T- and B-lymphoid cells and have compared the selectivity of this nucleoside with that of the related arabinonucleoside of cytosine.

Materials and Methods. *Nucleoside analogs.* ara-C was from the Upjohn Company, Kalamazoo, Michigan; ara-G, ara-GTP, and ara-CTP were from Calbiochem, La Jolla, California. Nucleosides were from Sigma Chemical Company, St. Louis, Missouri. [5,6-^{3}H]ara-C (Moravek Biochemicals, Inc., Brea,

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[2] Abbreviations used: ara-C, 1-β-D-arabinofuranosylcytosine; Gua, guanine; ara-G, 9-β-D-arabinofuranosylguanine; dAdo, 2′-deoxyadenosine; dCyd, 2′-deoxycytidine; dGuo, 2′-deoxyguanosine; Ado, adenosine; Guo, guanosine; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; IC_{50}, 50% inhibitory concentration; HPLC, high-performance liquid chromatography; dCF, deoxycoformycin.

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Calif.) was greater than 98% radiochemically pure and was used without further purification. [^{3}H]ara-G (Amersham Tritium Labeling Service) was routinely purified by reverse-phase chromatography on a Partisil-5 ODS column (4.6 × 250 mm, Whatman, Inc.), as previously described (7).

Cell lines and cell culture methods. The CCRF-CEM T-lymphoid leukemic cell line and a dCyd kinase-deficit subline (hereafter termed CEM and CEM/dCK$^-$) have been described (7). WI-L2 splenic B lymphoblasts and a dCyd kinase-deficient subline (WI-L2/dCK$^-$) were obtained from Dr. Michael S. Hershfield (Duke University, Durham, N.C.). RPMI 8402 (GM 3639), Molt-4 (GM 2219B), and RPMI 8392 (GM 3638) were from the Institute for Medical Research, Camden, New Jersey. The RAJI (CCI 86) line was from American Type Culture Collection, Rockville, Maryland. PF-2S B-lymphoid leukemic cells were obtained from Dr. Alan Tereba (St. Jude Children's Research Hospital, Memphis, Tenn.) and RPMI 6410 cells, previously described (8), were from Dr. Arnold Welch (St. Jude Children's Research Hospital).

In general all cell lines were cultured at 37°C in 75-cm^2 tissue culture flasks (Costar 3275, Bellco Glass, Inc., Vineland, N.J.) in a high humidity, 5% CO_2 atmosphere. For Molt-4, RPMI 8392, RAJI, PF-2S, and RPMI 6410, the culture medium was composed of RPMI 1640 medium supplemented with 2 m*M* glutamine (Grand Island Biological Co., Grand Island, N.Y.), sodium bicarbonate (2.2 g/liter), penicillin (60 μg/ml), streptomycin (100 μg/ml), and 10% heat-inactivated newborn calf serum (Flow Laboratories, Rockville, Md.). For RPMI 8402 and WI-L2 cultures, 10% heat-inactivated horse serum (Flow Laboratories) was used instead of newborn calf serum. Culture conditions for CEM cells have been described (7). Cell cultures and serum were tested by previously described methods (9) and found to be free of mycoplasma and adenosine phosphorylase activity.

Growth inhibition studies. Experiments to determine the effects of arabinonucleosides on T and B lymphoblasts were conducted with 25-cm^2 tissue culture flasks (Costar 3050). Small volumes (10–200 μl) of growth inhibitory agent were pipetted into each flask, after which 5 ml of cells (0.1 to 0.2 × 10^6 cells/ml) was added in complete medium. After 48 hr the number of untreated cells typically increased from four- to eightfold. The initial cell densities were subtracted from the final cell densities, and the number of cells in the flasks containing growth inhibitory arabinonucleoside was calculated as a percentage of the number of untreated cells in control flasks. The drug concentration inhibiting cell growth by 50% (IC_{50}) was determined from semilog graphs of percentage cell growth versus drug concentration.

Arabinonucleoside accumulation as arabinonucleoside 5'-triphosphate. The capacities of intact T and B lymphoblasts to accumulate arabinonucleoside 5'-triphosphate from extracellular arabinonucleoside were compared in the following manner. Aliquots of exponentially growing cells (25 ml) at a density of 0.4 to 0.8 × 10^6 cells/ml were incubated with 100 μM [^{3}H]ara-C (10 Ci/mole) or [^{3}H]ara-G (100 Ci/mole). Four hours after addition of [^{3}H]arabinonucleoside, cells were harvested by a 5-min centrifugation in 50-ml Falcon centrifuge tubes, the medium was decanted, and the pellets suspended in 0.25 ml of cold 0.5 *N* perchloric acid and transferred to 1.5-ml microfuge tubes. Ten minutes later the acid-insoluble precipitate was removed by centrifugation and the supernatant, containing the acid-soluble arabinonucleotides, was neutralized by addition of 12.5 μl 1 *M* potassium phosphate, pH 7.5, and 12.5 μl 10 *N* potassium hydroxide. The resulting potassium perchlorate was removed by centrifugation and the supernatant stored at −20°C until HPLC analysis.

On the day of analysis, the ribonucleotides were degraded by sodium periodate following the method of Garrett and Santi (10) and [^{3}H]arabinonucleoside 5'-triphosphates were quantitated by HPLC on a Partisil-10 SAX column (4.6 × 250 mm, Whatman, Inc.) using a mobile solvent of 0.4 *M* ammonium phosphate, pH 3.25 (pH 3.0 for ara-CTP), with 10% acetonitrile at a flow rate of 2 ml/min. Fractions were collected every 0.4 min and analyzed by liquid scintillation analysis. Each [^{3}H]arabinonucleoside 5'-triphosphate was identified by comparing of its retention time with a known standard.

Arabinonucleoside 5'-triphosphate degradation. The capacities of intact CEM, PF-2S, and WI-L2 cells to catabolize arabinonucleoside 5'-triphosphates were compared as follows. Exponentially growing CEM cells (150 ml, 0.4–0.8 × 10^6 cells/liter) were incubated with one of the following amounts of drug: 0.5 μM [^{3}H]ara-C (200 Ci/mole) or 10 μM [^{3}H]ara-G (50 Ci/mole). PF-2S cells were incubated in the same manner but with 1 μM [^{3}H]ara-C (200 Ci/mole), or 250 μM [^{3}H]ara-G (50 Ci/mole); WI-L2 cells were incubated with 5 μM [^{3}H]ara-C (100 Ci/mole) or 200 μM [^{3}H]ara-G (50 Ci/mole). Four hours later the cells were transferred to a large-volume glass centrifuge tube (Belco No. 3045-00600) cooled to 20°C and centrifuged at 1000*g* for 15 min. The medium was decanted and discarded and the pellet resuspended in 150 ml of fresh culture medium (20°C) without drug. Five 25-ml aliquots were then dispersed to 50-ml Falcon tubes, one of which was centrifuged immediately and the others incubated at 20°C (with periodic mixing) for 15, 30, 60, and 120 min before centrifuging. The medium was decanted and the cell pellets suspended in 0.25 ml 0.5 *N* perchloric acid and analyzed for arabinonucleoside 5'-triphosphate levels as indicated.

Results. *Growth inhibitory effects of arabinonucleosides.* The growth inhibitory effects of ara-G and ara-C on eight human T- and B-lymphoblast cell lines were compared. The initial cell densities selected for each cell line were necessary to ensure logarithmic growth for each. ara-G inhibited the growth of T lymphoblasts by about 100-fold more than B lymphoblasts (Fig. 1). By contrast, ara-C exhibited no selectivity for T- and B-cell lines. The selectivity of ara-G for T lymphoblasts is quantitatively similar to that seen with dAdo, in the presence of dCF (Fig. 1).

Degradation of ara-G. The ability of T and B cells to degrade ara-G was studied to determine whether or not this could be a basis for the selectivity of the compound. T (CEM) and B (WI-L2) lymphoblasts were incubated in culture medium containing 10 μM of radioactively labeled ara-G, and the disappearance of nucleoside from the cultured medium was monitored by HPLC. As shown

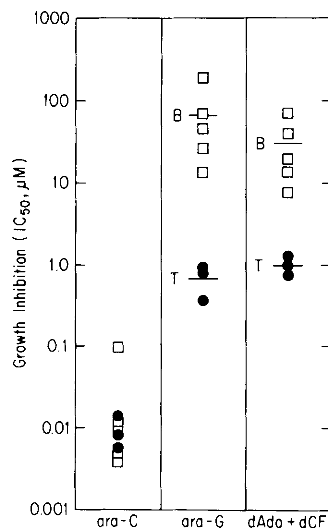

FIG. 1. Growth inhibitory activity of arabinonucleosides on T and B lymphoblasts. The concentration producing 50% inhibition of cell growth during 48 hr (IC_{50}) was determined for each compound and each cell line. Deoxyadenosine was tested in combination with nontoxic concentration of deoxycoformycin (3.8 μM) to prevent deamination. T lymphoblasts (●) = CCRF − CEM, Molt-4 and RPMI 8402; B lymphoblasts (□) = PF-2S, RAJI, RPMI 8392, RPMI 6410, WI-L2 (in order of increasing IC_{50}).

in Table I, at least 95% of the radioactivity in the media of both T and B cell lines was found with the nucleoside after a 48-hr incubation period. These results indicate that unlike its normal counterpart dGuo, ara-G is resistant to cleavage by PNP present in both T- and B-lymphoid cells.

Role of nucleoside kinase in ara-G activation. The growth inhibitory effects of ara-G on dCyd kinase-deficient mutants of CEM and WI-L2 cell lines were also compared. The IC_{50} concentration of ara-G in CEM mutants was 210 μM compared with 0.8 μM for the wild type, an increment of 240-fold (Fig. 2). In marked contrast, WI-L2 mutant cells lacking dCyd kinase activity exhibited only a twofold increase in IC_{50} value.

The kinase-deficient mutants were further characterized for their ability to metabolize ara-G to the nucleotide level. With 100 μM ara-G in the culture medium, CEM cells accumulated 80 pmole ara-GTP/10^6 cells after 4 hr of incubation, while the kinase-

TABLE I. ara-G DEGRADATION IN CEM AND WI-L2 CELLS

Cell type	Time (hr)	Distribution of radioactivity		
		ara-G	Gua	Other[a]
		(dpm/μl culture)		
CEM	0	3060	13	70
	24	2910	5	200
	48	2940	12	345
WI-L2	0	3260	13	70
	24	2630	60	160
	48	2400	128	255

Note. CEM or WI-L2 cells (0.2 × 10^6 cells/ml) were cultured with 10 μM [^{3}H]ara-G (100 Ci/mole) and aliquots were withdrawn at various times. Each aliquot was extracted with an equal volume of 0.5 *N* perchloric acid, and the acid-soluble material was removed by centrifugation. The supernatant was neutralized with 10 *N* KOH and the resulting potassium perchlorate precipitate removed by centrifugation. The ara-G and Gua in these samples were then separated by HPLC using a Whatman Partisil 5 ODS column eluted isocratically with 2.5 mM KH_2PO_4, pH 3.5, and 2.5% methanol at 1 ml/min. Fractions were collected at 0.5 min intervals and analyzed by liquid scintillation.

[a] Mainly tritiated water which eluted in the breakthrough region of the chromatogram.

deficient subline accumulated about 2.5 pmole ara-GTP/10^6 cells, approximately the same level as formed in WI-L2 wild-type cells and the kinase-deficient counterpart (Fig. 3).

Both cell lines were also examined for their ability to accumulate ara-CTP from ara-C

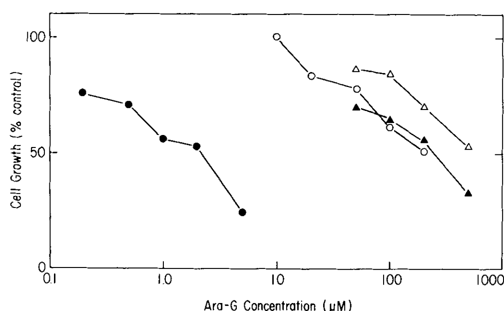

FIG. 2. Growth inhibition of wild-type and deoxycytidine kinase-deficient cells. The growth inhibition of various concentrations of ara-G was determined as described under Materials and Methods in wild-type CEM (●), dCK-deficient CEM (○), wild-type WI-L2 (▲), and dCK-deficient WI-L2 (△).

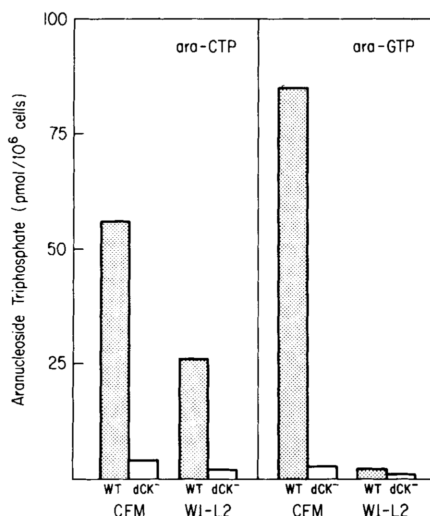

FIG. 3. Arabinonucleotide formation in CEM/dCK⁻ and WI-L2/dCK⁻ cells. Exponentially growing cells were incubated with 100 μM [^{3}H]ara-C or [^{3}H]ara-G at 37°C for 4 hr, and then the 5′-triphosphate metabolite was quantitated by HPLC as described under Materials and Methods.

(Fig. 3). The B cells (WI-L2) accumulated ara-CTP to approximately 50% of the level observed in the wild-type T cells (CEM). Kinase-deficient mutants accumulated less than 10% the amount of ara-CTP as their respective wild-type cells. Thus, the selectivity of ara-G for T-cell lines is associated with their increased ability to accumulate the nucleotides via dCyd kinase pathway.

It is unclear why ara-C and ara-G should differ so in their selectivity for T and B cells, as they apparently are phosphorylated by the same kinase pathway. One possibility is that the dCyd kinase has different specificity in T- and B-cell lines. This possibility seems unlikely, however, because dCyd kinase activity in CEM and WI-L2 extracts has similar affinities (apparent K_m values) for ara-G and ara-C.[3] Moreover, the ara-G phosphorylating activity in WI-L2 extracts was approximately five times higher than that of the more sensitive CEM cells (Table II). These findings do not support diminished kinase activity for ara-G in WI-L2 as compared with CEM cells

[3] Unpublished data.

TABLE II. PHOSPHORYLATING ACTIVITIES FOR ARABINONUCLEOSIDES IN WILD-TYPE AND DEOXYCYTIDINE KINASE-DEFICIENT LYMPHOBLASTS

	Arabinonucleoside phosphorylating activity (pmole/hr/mg protein)	
Cell type extract	ara-C	ara-G
CEM	2100	28
CEM/dCK$^-$	1	7
WI-L2	2000	140
WI-L2/dCK$^-$	1	33

Note. The phosphorylating activities of arabinonucleosides were measured by the procedures for analysis of dCyd kinase activity in lymphoblast extracts (7), using the following substrates: [^{3}H]ara-C (1 μM, 18 Ci/mmole), and [^{3}H]ara-G (1 μM, 1.2 Ci/mmole). Protein concentrations were determined by the Bio-Rad protein assay with bovine γ-globulin as a standard (Bio-Rad Laboratories, Richmond, Calif.).

as a basis for the inability of WI-L2 cells to accumulate ara-GTP.

An alternative possibility is that there is a difference in catabolism of the arabinonucleoside triphosphate in these cell lines. Indeed, when the degradation of ara-GTP and ara-CTP were compared, significant differences in their catabolic rates were noted. As shown in Fig. 4, accumulated ara-CTP in B cells decreased with a $t\frac{1}{2}$ of about 2.6 hr (PF-2S) and 7 hr (WI-L2) while that of ara-GTP was 0.8 hr (PF-2S) and 2.6 hr (WI-L2). Degradation was attributed to dephosphorylation of the triphosphates, since it was accompanied by the simultaneous increase of radioactive nucleoside in the medium (data not shown). By contrast, no degradation of either ara-CTP or ara-GTP was detected in T cells.

Discussion. We have demonstrated a difference between cytotoxic arabinonucleosides in terms of their selectivity for human lymphoblastoid cells. Many studies have been made of the mechanism for the differential sensitivity of T and B cells to naturally occurring nucleosides such as dAdo, Ado, dGuo, and Guo (3–6, 11), the impetus being the suspected lymphotoxic role of triphosphate accumulation in contributing to the lymphocyte dysfunction in adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiency in humans (12). We have focused on the arabinonucleosides because of their wide clinical potential as antiviral and antineoplastic agents.

The differences in metabolism of ara-G by T and B cells were unexpected findings. The dCyd kinase-deficient CEM cells are clearly accumulating fewer ara-G nucleotides than are wild-type cells and are 240-fold more cross-resistant to ara-G. The failure of dCyd kinase-deficient cells to accumulate ara-GTP from ara-G, therefore, establishes this enzyme as the functional ara-G phosphorylating activity in these cells. Despite equal kinase activities in cell-free extracts, the sensitivity of WI-L2 and CEM cells differed by more

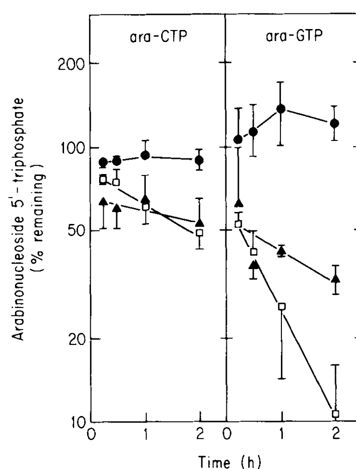

FIG. 4. Retention of arabinonucleotides in T cells and B cells. Exponentially growing CEM cells (●), PF-2S cells (□), or WI-L2 cells (▲) were allowed to accumulate arabinonucleoside 5'-triphosphates and then cooled to 20°C, centrifuged, and resuspended in fresh culture medium without drug. Aliquots of cells were removed immediately and at various time intervals thereafter and the level of intracellular 5'-triphosphate determined as described under Materials and Methods. The results are expressed as the percentage of 5'-triphosphate level determined immediately after the cells were resuspended (time 0). Vertical bars represent the standard deviation for three separate experiments. The data points for PF-2S and WI-L2 cells are fitted to a straight line by the method of least squares. Average arabinonucleotide levels at time 0 for ara-CTP were 70, 120, and 125 pmole/10^6 cells (CEM, PF-2S, WI-L2 cells, respectively); average levels at time 0 for ara-GTP were 14, 8.6, and 3.9 pmole/10^6 cells (CEM, PF-2S, WI-L2 cells, respectively).

than 250-fold. This difference in sensitivity was reflected by a 20- to 40-fold difference in the rate of ara-GTP accumulation between wild-type CEM and WI-L2 cells. A similar discrepancy between dAdo phosphorylating activity in cell-free extracts and dAdo nucleotide accumulation in intact cells was reported recently for CEM and WI-L2 (13, 14). It was suggested that in B lymphoblasts, dCyd kinase might be suppressed in its capacity to function as a purine phosphorylating enzyme and that a correlation may exist between sensitivity to purines in human lymphoid cells and the functional activity of dCyd kinase (14). We have found that substantial phosphorylation of the purine arabinonucleoside ara-A and its 2-fluoro derivative was catalyzed via dCyd kinase by intact CEM and WI-L2 cells[3] suggesting that, in both cell lines, dCyd kinase is operating as a purine phosphorylating enzyme.

It is possible, however, that inefficient accumulation of ara-GTP in B cells results from T-cell- and B-cell-specific isozymes of dGuo-phosphorylating enzymes. A comparison of analog-phosphorylating activities in extracts of CEM and WI-L2 cells shows about a fivefold higher level of ara-G phosphorylation and a twofold higher level of dGuo phosphorylation in WI-L2 cells, but similar levels of ara-C phosphorylating activities (Table II). These results suggest that there may be differences in the substrate specificity of dGuo kinases in T cells versus B cells. Although the high activity for ara-G phosphorylation in WI-L2 cell extracts appears contradictory to the reduced accumulation of ara-GTP in these cells, the B-cell enzyme activities may also be more sensitive to intracellular inhibitors than are the T-cell specific counterparts. Further studies are needed to clarify this point.

An alternative explanation for reduced accumulation of ara-GTP in the B cells could be related to the higher rate of catabolism of this arabinonucleotide in B cells versus T cells. The half-life of ara-GTP accumulated in B cells was approximately 0.8 hr (PF-2S) and 2.6 hr (WI-L2) at 20°C when ara-G was removed from the medium, whereas under similar conditions the level of analog nucleotide remained unchanged in T cells. Our results can be explained, therefore, by assuming that the B cells have a higher rate of arabinonucleotide-catabolizing activity, which limits accumulation of the nucleotide despite a high rate of ara-G phosphorylation. Furthermore, the high rate of ara-G nucleotide turnover relative to ara-CTP, indicates that the former is a preferred substrate for the catabolizing enzyme pathway(s) in B cells.

The enzymes involved in nucleotide catabolism in human lymphoid cells have remained controversial. Recently, Carson *et al.* (15) reported that human dAdo-resistant B lymphoblasts have a higher level of an intracellular nucleotidase activity than do the more sensitive T leukemic cells. This intracellular enzyme activity could be distinguished from a previously studied ecto-5′-nucleotidase by its preference for deoxyribonucleotides rather than ribonucleotides (15–17). However, as with assays of kinase activities, the difference in the activity, measured with dAMP as substrate, between T and B lymphoblasts (and specifically between CEM and WI-L2 cells) was only about threefold, insufficient to account for a 40- to 60-fold difference in rate of dATP accumulation between these cells (13). The expected pathway for conversion of nucleoside triphosphates to nucleotide is as follows: NTP $\rightarrow$ NDP $\rightarrow$ NMP $\rightarrow$ nucleoside. Thus far, only catabolism at the monophosphate level has been studied in some detail. However, metabolism must be examined beyond the monophosphate level to determine if this is the major limiting step for catabolism of triphosphates in cells. Further studies of arabinonucleoside triphosphate catabolism in intact cells and the isolation of mutants deficient in nucleotidase activities will increase our understanding of the mechanism(s) involved.

The selective effect of ara-G on T-lymphoblasts may have important therapeutic implications. Deoxyguanosine has been shown to selectively inhibit suppressor T-cell subpopulations (18) and to ablate thymocytes before thymic transplant (19). A major limitation of dGuo has been its rapid degradation to guanine by purine nucleoside phosphorylase present in erythrocytes. The availability of compounds, such as ara-G, that are resis-

tant to degradation but are similarly selective for T cells, could be of significant use for the treatment of human T-cell leukemias and for selective immunosuppression.

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