Metabolic Basis of Arabinonucleoside Selectivity for Human Leukemic T- and B-Lymphoblasts

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ABSTRACT

Purine analogues are potentially useful agents for selective chemotherapy of lymphoproliferative diseases. We compared the toxic effects of various arabinonucleosides against eight human T- and B-lymphoblastoid lines. The arabinosides of cytosine (ara-C), 2-fluorodeoxycytidine (F-ara-A), adenine (ara-A), and guanine (ara-G) all inhibited the growth of T-lymphoblasts at concentrations below 2 μM. Only ara-G showed strong selectivity for T-cells, as indicated by a 15- to 250-fold greater toxicity toward T-cell lines than B-cell lines. To investigate the biochemical basis for ara-G selectivity, we compared the metabolism of the arabinonucleosides in CCRF-CEM (T-) versus PF-2S (B-) lymphoblasts. Comparison of arabinonucleoside triphosphate accumulation indicated differences favoring selective ara-GTP formation in T-cells. In contrast, ara-C, ara-A, and F-ara-A formed almost corresponding amounts of their triphosphates in both cell types. Triphosphate accumulation correlated directly with inhibition of DNA synthesis in CCRF-CEM and PF-2S cells. PF-2S cells accumulated less than 20% ara-GTP from the nucleoside than did CCRF-CEM cells. Nucleoside kinase measurements showed no significant differences in arabinonucleoside phosphorylation that could account for the preferential ara-GTP accumulation in T-cells. After removal of arabinonucleoside-containing medium, ara-GTP levels in PF-2S cells declined with a half-life of 49 min whereas, in CCRF-CEM cells, the level of analogue triphosphate remained unchanged. Furthermore, the half-life of ara-CTP, ara-ATP, and F-ara-ATP in the B-cells was 3- to 5-fold longer than that of ara-GTP. These results indicate that ara-G is more selective than other known arabinonucleosides; such selectivity warrants further assessment of the therapeutic potential of this agent against T-cell malignancies and other lymphoid disorders.

INTRODUCTION

Purine deoxyarabinonucleosides can selectively alter lymphocyte proliferation and function. Inherited abnormalities in dAdo3 and dGuo metabolism have been associated with selective depletion of lymphoid cells in humans. Adenosine deaminase deficiency results in severe combined immune deficiency disease, characterized by a loss of both T- and B-lymphocytes, while purine nucleoside phosphorylase deficiency results in a selective T-cell deficit (1, 2). In both diseases, lymphocytotoxicity appears to result from accumulation in plasma of 2’-deoxyribonucleoside substrates for these 2 enzymes: dAdo for adenosine deaminase deficiency and dGuo for purine nucleoside phosphorylase deficiency (3). In affected persons, T-lymphocytes may selectively metabolize the deoxyribonucleosides, which are excreted by other tissues that are unable to retain the active deoxyribonucleoside triphosphates (4–6). These findings have stimulated interest in the potential use of purine analogues for selective immunosuppression and treatment of malignant lymphoproliferative diseases. Previous experimental approaches to this question have focused on the use of adenosine deaminase inhibitors such as 2’-deoxycoformycin. However, administration of this compound to patients with leukemia has resulted not only in malignant cell reduction but also in severe toxicity to lungs, kidneys, and central nervous system (7).

Recently, Cohen et al. (8) identified a deoxyguanosine derivative, ara-G, that was selectively toxic to human T-lymphoblastoid cells at micromolar concentration. It was resistant to phosphorolysis by purine nucleoside phosphorylase and was actively converted to the presumed active triphosphate form. The goals of our investigations were: (a) to compare, in human T- and B-lymphoblastoid lines, the toxicity and metabolism of various arabinonucleosides, including ara-C, ara-A, the adenosine deaminase-resistant analogue 2-fluoro-ara-A, and ara-G; and (b) to ascertain the relative roles of arabinonucleoside phosphorylation and triphosphate analogue accumulation in mediating any observed drug selectivity. Some of our findings are described in a preliminary report (9).

MATERIALS AND METHODS

Nucleoside Analogues. ara-C was purchased from the Upjohn Co., Kalamazoo, MI; ara-A, F-ara-A, and deoxycoformycin were from the National Cancer Institute, Bethesda, MD; and ara-G, ara-GTP, ara-ATP, and ara-GTP were from Calbiochem-Behring Corp., La Jolla, CA. Nucleosides were from Sigma Chemical Co., St. Louis, MO; [methyl-3H]dThd was from Schwarz/Mann, Inc., Spring Valley, NY. The radioactively labeled analogues [8-3H]F-ara-A and [5,6-3H]ara-C (Moravek Biochemicals, Inc., Brea, CA) were all more than 98% radiochemically pure and were used without further purification. [2,8-3H]ara-A (Moravek Biochemicals) and [5H]ara-G (Amersham Trilabeling Service) were purified routinely by reversed-phase chromatography on a Partisil-5 ODS column (4.6 x 250 mm, Whatman, Inc.) as described previously (10).

Cell Lines and Cell Culture Methods. The CCRF-CEM T-lymphoid leukemic cell line (hereafter termed CEM) has been described (11) and was obtained from the American Type Culture Collection, Rockville, MD. PF-2S B-lymphoid leukemia cells were obtained from Dr. Alan Tereba (St. Jude Children’s Research Hospital, Memphis, TN), and RPMI 6410 cells (12) were from Dr. Arnold Welch (St. Jude Children’s Research Hospital, Memphis, TN).
In general, all cell lines were cultured at 37°C in 75-cm tissue culture flasks (Costar 3275, Belco Glass, Inc., Vineland, NJ) with high humidity and 5% CO₂ in air. For Mol-4, RPMI 8392, RAJI, PF-2S, and RPMI 6410, the culture medium was composed of RPMI 1640 medium supplemented with 2 mM glutamine (Grand Island Biological Co., Grand Island, NY), 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 the newborn calf serum. Culture conditions for CEM cells have been described (10).

Cell cultures and serum were tested and found to be free of Mycoplasma and adenosine phosphorylase activity by methods described previously (13). The average cell volume of exponentially growing cells was determined by a Coulter Counter (Model Z) calibrated with monodispersed latex particles.

Growth Inhibition Studies. Experiments to determine the effects of arabinonucleosides on T- and B-lymphoblasts were conducted in 25-cm tissue culture flasks (Costar 3050). Small volumes (10 to 200 μl) of growth-inhibitory agent were pipetted into each flask, after which 5 ml of cells (0.1 to 2 x 10⁶ cells/ml) in complete medium was added. After 48 h, the number of untreated cells typically increased from 4- to 8-fold. The initial cell densities were subtracted from the final cell densities, and the number of cells in the flasks containing the growth-inhibitory arabinonucleoside was calculated as a percentage of the number of untreated cells in control flasks. The IC₅₀ was determined from semi-log graphs of percentage of cell growth versus drug concentration.

DNA Synthesis in Drug-treated Lymphoblasts. Cells were incubated in culture media for 4 h with or without an equitoxic concentration (IC₅₀) of each of the 4 arabinonucleosides and pulsed with [³H]dThd (0.5 μM, 0.3 Ci/mmol) for an additional 15 min. The cells were harvested and extracted with 0.5 N perchloric acid as described below. The acid-insoluble precipitate that contained the DNA was solubilized with NCS (Amersham) and counted for radioactivity. The acid-soluble extract that contained [³H]dThd nucleotides was neutralized, digested with sodium periodate, and analyzed by HPLC (see below). The TTP in these samples was detected by UV absorption at 254 nm and quantitated by comparing the peak heights of the unknown levels of TTP in each sample with peak heights of known amounts of standard TTP. In addition, TTP eluting from the HPLC column was collected and analyzed for radioactivity. Rates of DNA synthesis were calculated for drug-treated and untreated cells from the specific activity (dpm/pmol) of the [³H]dThd and the amount of radioactivity incorporated into DNA per million cells.

Arabinonucleoside 5'-Triphosphate Accumulation. The capacities of intact T- and B-lymphoblasts to accumulate arabinonucleoside 5'-triphosphate from extracellular arabinonucleoside were compared in the following manner. Twenty-five-ml aliquots of exponentially growing cells at a density of 0.4 to 0.8 x 10⁶ cells/ml were incubated with 10-fold incremental doses of radioactively labeled arabinonucleoside. For [³H]ara-C, the concentration range was 0.01 μM (1000 Ci/mmol) to 100 μM (10 Ci/mmol); for [³H]ara-A, it was 0.1 μM (5000 Ci/mmol) to 100 μM (5 Ci/mmol); for [³H]ara-G, it was 0.1 μM (10,000 Ci/mmol) to 100 μM (10 Ci/mmol); and for [³H]ara-A, it was 0.1 μM (10,000 Ci/mmol) to 100 μM (10 Ci/mmol). Four h after addition of tritiated arabinonucleoside, the cells were harvested by a 5-min centrifugation in 50-ml Falcon centrifuge tubes, the medium was decanted, and the pellets were resuspended in 0.25 ml of 0.5 N perchloric acid and analyzed by HPLC as indicated.

RESULTS

Growth Inhibition. IC₅₀ values for the 4 arabinonucleosides, studied with 8 human lymphoblastoid lines, are shown in Table 1. ara-C, ara-A (in the presence of the adenosine deaminase inhibitor 2'-deoxycoformycin), F-ara-A, and ara-G were all markedly cytotoxic to T-lymphoblasts at concentrations below 2 μM. Only ara-G showed strong selectivity for T-cells. Some variation in the degree of resistance among B-cell lines was observed, but the most sensitive cell line, PF-2S, was at least 15-fold more resistant to ara-G than were any of the 3 leukemic T-cell lines CEM, 8402, or Mol-4. These results are comparable to those reported by Cohen et al. (8). By contrast, ara-C and F-ara-A were weakly selective, showing less toxicity to only 2 B-cell lines, RPMI 6410 and WI-L2, compared with the T-cell lines. ara-A with deoxycoformycin showed intermediate selectivity.

Arabinonucleoside Triphosphate. The intracellular levels of 10 SAX column (4.6 x 250 mm; Whatman, Inc.). A mobile solvent consisting 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 was used. Fractions were collected every 0.4 min and analyzed by liquid scintillation. Each tritiated arabinonucleoside 5'-triphosphate was identified by comparing its retention time with a known standard.

Arabinonucleoside 5'-Triphosphate Degradation. The capacities of intact CEM and PF-2S cells to catabolize arabinonucleoside 5'-triphosphates were compared. Exponentially growing CEM cells (150 μl, 0.4 to 0.8 x 10⁶ cells/ml) were incubated with a 25-cm tissue culture flask and 0.1 MM [³H]ara-G, 0.8 MM [³H]ara-A (160 Ci/mol) plus 4 μM deoxycoformycin, or 10 μM [³H]ara-G (50 Ci/mol). PF-2S cells were incubated in the same fashion but with 1 μM [³H]ara-C (200 Ci/mol), 4 μM [³H]ara-A (55 Ci/mol), 25 μM [³H]ara-A (160 Ci/mol) plus 4 μM deoxycoformycin, or 250 μM [³H]ara-G (50 Ci/mol). Four h later, the cells were transferred to a large volume glass centrifuge tube (Belco No. 3045-00600), cooled to 20°C, and centrifuged at 1000 x g for 15 min. The medium was decanted and discarded, and the pellet was 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. The others were incubated at 20°C (with periodic mixing) for 15, 30, 60, or 120 min before centrifuging. The medium was decanted, and the cell pellets were resuspended in 0.25 ml of 0.5 N perchloric acid and analyzed for arabinonucleoside 5'-triphosphate levels as indicated.

Table 1

<table>
<thead>
<tr>
<th>Drug concentration (μM) inhibiting growth by 50%</th>
<th>ara-A + 4 μM deCoF</th>
<th>ara-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara-C</td>
<td>0.014</td>
<td>1.6</td>
</tr>
<tr>
<td>F-ara-A</td>
<td>0.006</td>
<td>0.8</td>
</tr>
<tr>
<td>ara-A</td>
<td>0.008</td>
<td>1.4</td>
</tr>
<tr>
<td>ara-G</td>
<td>0.006</td>
<td>0.8</td>
</tr>
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</table>

* deCoF (deoxycoformycin) was added to cells 30 min before addition of ara-A. Control experiments showed that deoxycoformycin alone had no effect on cell growth under these conditions.

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the 4 arabinonucleoside triphosphates formed by incubation of the analogues with the CEM T-cell and PF-2S B-cell lines are shown in Chart 1. In the presence of as little as 1 μM ara-G, CEM accumulated approximately 5-fold more ara-GTP than did the B-cell line and 20-fold more when 100 μM nucleoside was added. For ara-C and F-ara-A, incubation of the cells with increasing concentrations resulted in no more than a 2-fold difference in the levels of the corresponding triphosphates between T- and B-cell lines and, for ara-A with deoxycoformycin, no more than a 4-fold difference. Assuming a uniform intracellular distribution and using the mean cell volumes of 1.42 and 1.08 μl per 10⁶ cells for CEM and PF-2S cells, we determined that, at equitoxic concentrations for each analogue, the cellular level of ara-GTP was about 1 μM; of ara-ATP, it was 2 to 3 μM; of ara-CTP, it was 0.4 to 0.8 μM; and of F-ara-ATP, 5 to 10 μM (Table 2). Under these conditions, DNA synthesis was inhibited 60 to 90% for both cell types (Table 2). These data suggest that the sensitivity of the intracellular target for all of these analogues is similar in CEM versus PF-2S lymphoblasts; thus, their selectivity is determined mainly at the level of nucleotide uptake and retention.

**Arabinonucleoside Phosphorylating Activities.** Table 3 compares the levels of nucleoside analogue phosphorylating activities in intact cells and nucleotide analogue accumulation in intact cells for CEM and PF-2S lymphoblasts. Surprisingly, all 4 arabinonucleosides, including the T-cell-selective agent ara-G, were phosphorylated 50 to 250% faster by B-lymphoblast extracts compared with T-lymphoblast extracts. The results also indicate that the analogue phosphorylating activities in extracts are as much as 94-fold greater than the rates of accumulation of the corresponding analogue 5′-triphosphates in intact cells. The suppression of arabinonucleotide accumulation in CEM cells (calculated as the rate of analogue phosphorylation in extracts divided by the rate of analogue 5′-triphosphate accumulation in intact cells; see Table 3) is most evident for ara-CTP while, in PF-2S cells, the suppression is greatest for ara-GTP. The higher levels of nucleoside analogue phosphorylating activities in PF-2S extracts are consistent with the higher levels of ara-CTP and F-ara-ATP accumulation by intact PF-2S cells, but they do not explain the apparent suppression of ara-ATP and ara-GTP accumulation in these cells compared with the CEM line.

**Dephosphorylation of Triphosphates.** We next investigated the ability of the CEM and PF-2S cell lines to degrade accumulated arabinonucleotides. The approach was to incubate the cells with radioactive compound for 4 h to achieve a range of intracellular concentration of labeled nucleotides and then, after reincubation of the cells at 20°C in fresh medium free of radioactivity, to measure the loss of radioactivity from intracellular pools by HPLC analysis. At 20°C, the rate of incorporation of radioactivity into nucleic acids is minimal, permitting a more accurate comparison of nucleotide degradation. In addition, we observed that the rate of degradation of the nucleotide pools is about one-fourth the rate at 37°C; thus, longer time courses can be used at higher effective specific radioactivity. As shown in Chart 2, degradation of the nucleotide analogues followed first-order kinetics and progressed linearly during the time course studied. The half-life in PF-2S was estimated to be 49 min for ara-GTP, 190 min for ara-ATP, 370 min for F-ara-ATP, and 160 min for ara-CTP. Degradation was assumed to be due to dephosphorylation of the triphosphate, as it was accompanied by the simultaneous increase of radioactive nucleoside in the medium (data not shown). By contrast, no degradation of any of the arabinonucleotides could be detected in the CEM cells under these conditions.

**DISCUSSION**

We have demonstrated differences among cytotoxic arabinonucleosides in terms of their selectivity for human lymphoblastoid...
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Chart 2. Retention of arabinonucleotides in T- and B-lymphoblasts. Exponentially growing CEM cells (○) or PF-2S cells (△) were allowed to accumulate high levels of arabinonucleoside 5'-triphosphates and were then cooled to 20°C, centrifuged, and resuspended in fresh culture medium without drug. Aliquots of cells were removed at 0, 1, 2, and 3 h, and the level of intracellular 5'-triphosphate was determined. 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 3 separate experiments. The data points for PF-2S cells are fitted to a straight line by the method of least squares. Average arabinonucleotide levels at Time 0 for ara-CTP, F-ara-ATP, ara-ATP, and ara-GTP were 70, 59, 45, and 14 pmol/million cells, respectively, for CEM cells and 120, 110, 78, and 8.6 pmol/million cells for PF-2S cells.

Cells. Many studies have been done of the mechanism for the differential sensitivity of T- and B-cells to the naturally occurring nucleosides such as dAdo, adenosine, dGluo, and guanosine (15-20), the impetus for such research being the suspected lymphotoxic roles of triphosphate accumulation in contributing to the lymphocyte dysfunction in adenosine deaminase and purine nucleoside phosphorylase deficiency in humans (3, 5). We have focused on the arabinonucleosides because of their clinical potential as antiviral and antineoplastic agents.

The 4 arabinonucleosides, ara-A, F-ara-A, ara-G and ara-C, showed various degrees of selectivity toward human lymphoblast lines. Human malignant T-lymphoblasts were 15- to 250-fold more sensitive to the toxic effect of the purine nucleoside phosphorylase-resistant dGluo analogue ara-G than were human B-lymphoblasts. Conversely, the sensitivity of these different cell lines to the toxic effect of the other 3 arabinonucleosides differed by not more than 10-fold. This difference in sensitivity was reflected by a 5- to 20-fold lower rate of ara-GTP accumulation in the leukemic B-cell line PF-2S versus the T-cell line CEM. Nonetheless, the data are not consistent with the 2-fold higher levels of arabinonucleoside phosphorylating activities in the PF-2S extracts (Table 3). We showed previously that, in CEM cells, the toxic effect of ara-G, F-ara-A, and ara-A with an adenosine deaminase inhibitor requires phosphorylation by dCyd kinase at the lowest concentration at which these drugs are active (10, 20, 22). Adenosine kinase also acts to phosphorylate ara-A but not F-ara-A or ara-G, but mainly when the nucleoside concentration is raised above 100 μM (10). The lack of an important difference in nucleoside kinase activity between PF-2S and CEM cells is confirmed, therefore, by the comparable conversion of the arabinonucleosides ara-C, ara-A, and F-ara-A to trinucleosides in both lymphoblast types.

A similar discrepancy between dAdo phosphorylating activity in cell-free extracts and dAdo nucleotide accumulation in the intact cells was reported recently for CEM and WI-L2 (23, 24). It was suggested that, in WI-L2 B-lymphoblasts, the capacity of dCyd kinase to function as a purine phosphorylating enzyme is suppressed and that a correlation exists between sensitivity to purines in human lymphoid cells and the functional activity of dCyd kinase (24). The substantial accumulation and toxicity of ara-C and the purines F-ara-A and ara-A in intact CEM and PF-2S cells (Table 1 and Chart 1) suggests, however, that in both cell lines, dCyd kinase is operating as a pyrimidine and purine phosphorylating enzyme.

The differential incorporation of ara-G in PF-2S versus CEM cells may depend more on the rate of ara-GTP catabolism. The half-life of ara-GTP accumulated in B-cells was approximately 49 min at 20°C, whereas, in similar conditions, the analogue nucleotide level remained unchanged in T-cells. In addition, the high rate of ara-G nucleotide turnover relative to ara-ATP, F-ara-ATP, and ara-CTP (Fig. 2) correlates with a relatively high suppression of ara-GTP accumulation compared with the other analogues in PF-2S cells (Table 3). One possible explanation for the relative resistance to ara-G is that PF-2S cells have high rates of catalyzing activity that preferentially limit ara-GTP accumulation, despite high levels of phosphorylating activity.

Arabinonucleotide turnover in T-lymphoblasts, although undetectable at 20°C, is evident at 37°C when measured for extended periods of time. Plunkett et al. (26) reported that the half-life of F-ara-ATP in CEM cells is 2.5 h, and Abe et al. (25) reported a similar half-life for ara-CTP in Mol-4F cells. In those cells, however, ara-GTP and ara-ATP are retained much longer, having half-lives of about 15 h (26). Thus, the selective accumulation and toxicity of ara-A and, more importantly, of ara-G not only depends on the high rate of nucleotide turnover in B-cells but also on the unique stability of the triphosphates of these analogues in T-cells.

The potent action of ara-G against T-lymphoblasts (IC50 < 1 μM; Table 1), together with the resistance of B-lymphoblasts to this agent, are important theoretical considerations when considering the therapeutic use of ara-G for T-cell diseases and for selective immunosuppression. Our studies show that ara-C, F-ara-A, and ara-A, in the presence of deoxycoformycin, although active against human lymphoid and myeloid leukemias, are much less selective for lymphoid subtypes. Moreover, deoxycoformycin alone, which acts in part by causing deoxyadenosine accumulation, appears to have limited usefulness as a selective chemotherapeutic agent, probably because of the accumulation of nonselective toxic metabolites (7). ara-G, conversely, has the same magnitude of selectivity for T-lymphoblasts as do deoxyadenosine and deoxyguanosine (8, 20). These observations are especially significant, as deoxyguanosine has been shown to selectively inhibit suppressor T-cell subpopulations (27) and to ablate thymocytes before thymic transplant (28).

A major limitation of deoxyguanosine as a selective agent has been its rapid degradation to guanine by purine nucleoside phosphorylase present in erythrocytes. The availability of compounds such as ara-G, which are resistant to degradation yet are readily phosphorylated to their active derivatives in lymphoid tissues, offers a rational approach for the selective treatment of human lymphoid cancers or other T-cell disorders, such as autoimmune disease.

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* Unpublished data.
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