Specific Cytotoxicity of Arabinosyl-guanine toward Cultured T Lymphoblasts

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Abstract. Purine nucleoside phosphorylase (PNP) deficiency in humans is associated with a severe T cell immunodeficiency. To understand further and exploit this T cell lymphospecificity, we have compared the cytotoxicities and metabolism of deoxyguanosine, the cytotoxic substrate of PNP and of arabinosylguanine, a deoxyguanosine analogue that is resistant to PNP cleavage, in T cell (8402) and B cell (8392) lines in continuous culture established from the same patient. In comparative growth rate experiments the T cells were 2.3-fold and 400-fold more sensitive to growth inhibition by deoxyguanosine and arabinosylguanine, respectively, than were the B cells. Only the T cells, but not the B cells, could phosphorylate in situ deoxyguanosine or arabinosylguanine to the corresponding triphosphate. Both the phosphorylation and cytotoxicity of arabinosylguanine in the T cell line could be prevented by deoxycytidine, which suggests that deoxycytidine-deoxyguanosine kinase initiated the intracellular metabolism and cytotoxicity of this nucleoside analogue. The sensitivity and selectivity of arabinosylguanine toward the T lymphoblastoid cells suggests a rational approach to the design of chemotherapeutic agents that are directed toward T cell malignancies and other T cell disorders.

Introduction

Biochemical and genetic analyses of the pathogenic mechanisms by which a deficiency in purine nucleoside phosphorylase (PNP)[1] causes a selective T cell immunodeficiency (1) have provided important insights into the critical role of this enzyme to normal immune function. Cell culture models with murine

T cells that are genetically deficient in PNP have suggested that deoxyguanosine is the only PNP substrate which causes significant cytotoxicity (2). Coupled with the observation that deoxyguanosine triphosphate (dGTP) accumulates in the erythrocytes of PNP-deficient children (3) and in thymocytes that are exposed to deoxyguanosine (4), it appears that deoxyguanosine is the cytotoxic substrate of PNP. In order to exert its lymphotoxic effect, deoxyguanosine requires intracellular phosphorylation to the triphosphate level (5–9). dGTP inhibits the cytidine diphosphate reduction component of ribonucleotide reductase, depleting intracellular deoxycytidine triphosphate (dCTP) pools to levels inadequate for the maintenance of DNA synthesis (10). Cells that contain a ribonucleotide reductase activity that is refractory to complete inhibition by dGTP (2) and nonreplicating peripheral blood lymphocytes (11) are not sensitive to deoxyguanosine toxicity. The selective cytotoxicity of deoxyguanosine toward T cells appears to be the result of the unique ability of T cells to accumulate dGTP from exogenous deoxyguanosine (6–9). T cells have both the highest levels of deoxycytidine-deoxyguanosine kinase activity (12) and the lowest amounts of cytosolic deoxyribonucleotide dephosphorylating activity (13). The selectivity of PNP deficiency for cells that are derived from the T limb of the immune system offers a rational approach for the design of chemotherapies that are directed against either neoplasms of T cell origin or other T cell disorders.

In this report, we examined the metabolism and cytotoxicity of arabinosylguanine, a PNP-resistant deoxyguanosine analogue (14), in established T lymphoblastoid and B lymphoblastoid cell lines that were derived from the same individual. The potential for arabinosylguanine or similar analogues for the treatment of T cell lymphoproliferative diseases or disorders of T cell function is discussed.

Methods

Chemicals. Arabinosylguanine was a generous gift from Dr. Thomas Krenitsky of the Burroughs Wellcome Foundation, Research Triangle Park, NC. Drugs 5-amino-3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine-7-thione, 5-amino-3-β-D-arabinofuranosylpyrazolo[4,3-*d*]pyrimidine-7-thione, and 5-amino-3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrazolo[4,3 - *d*]pyrimidine - 7 -thione were provided by Dr. Edward Acton of SRI International, Menlo Park, CA. All of the materials, chemicals, and reagents were of the highest qualities commercially available.

Cell culture. The human T lymphoblastoid cell line, 8402, and the B lymphoblastoid cell line, 8392, were initially derived from the same patient (15, 16) and generously provided by Dr. Hans Ochs at the University of Washington Medical Center, Seattle, WA. Thus, both cell lines are theoretically syngeneic, except for the presumed Epstein Barr virus sequences in the B lymphoblastoid cells. Both cell lines were grown in RPMI 1640 medium that contained 10% heat inactivated (56°C, 30 min) fetal calf serum in a humidified 5% CO_2 incubator.

Growth experiments. The sensitivities of 8402 and 8392 to growth inhibition by cytotoxic agents were ascertained in Costar multi-well (24 well) tissue culture plates (Costar, Cambridge, MA). Small volumes (10–40 μl) of deoxyguanosine, arabinosylguanine, or other agent, were added to 10^5 cells in 1.0 ml of growth medium. After 96 h, cells were enumerated in a Coulter counter, model ZB1, Coulter Electronics, Inc., Hialeah, FL. This period of growth was chosen since both the 8402 and 8392 cell lines in control wells lacking agent grew exponentially over this period of time, approximately four cell divisions. The initial cell density was subtracted from the final cell density, and the resulting difference was plotted as a percentage of the growth of cells in control wells lacking agent as a function of the concentration of deoxyguanosine or arabinosylguanine.

Intracellular nucleotide measurements. 8402 and 8392 cells were grown overnight in complete medium to assure asynchronous and exponential growth. To 20-ml cell cultures that contained ~6 × 10⁵ cells/ml was added either 5–500 μM deoxyguanosine or 100 μM arabinosylguanine. Some cultures also contained various concentrations of deoxycytidine. Two flasks of each cell type that lacked nucleoside were maintained as controls. After 4-h incubations at 37°C under normal growth conditions, cells were pelleted by centrifugation for 8 min at 1,000 *g*, resuspended in 1.0 ml of phosphate-buffered saline, and centrifuged at 10,000 *g* for 6 s in a Microfuge B (Beckman Instruments, St. Louis, MO). A small aliquant of cells was removed for cell counting, and the nucleotides were extracted from the cell pellets with 1.5 N perchloric acid in 0.1 M phosphate, followed by neutralization with 1.0 N KOH (17). Separation of GTP, dGTP, and arabinosylGTP was performed on a high performance liquid chromatography system using a Whatman partisil SAX-10 column with 0.4 M ammonium phosphate buffer, pH 3.5, as the mobile phase at a flow rate of 1.5 ml/min. The retention volumes in this liquid system for GTP, dGTP, and arabinosylGTP were 42, 48, and 54 ml, respectively. The identification of intracellular GTP, dGTP, and arabinosylGTP could be ascertained by comparison of ratios of optical density at 254 and 280 nm and co-migration with known nucleotide standards. GTP and dGTP could be quantitated by comparisons of peak heights with those of standards, and arabinosylGTP measured by weighing the peaks recorded on the chart paper and comparing with the weights of the peaks of GTP and dGTP standards.

Results

Purine nucleoside phosphorylase deficiency results in a specific T cell immunodeficiency (1). Since deoxyguanosine is the only cytotoxic substrate of PNP (2, 5) and accumulates as dGTP in erythrocytes of PNP-deficient children (3), the growth inhibitory effects of exogenous deoxyguanosine were compared on the syngeneic 8402 T and the 8392 B cell lines. As shown in Fig. 1, the effective concentrations of deoxyguanosine which inhibited growth by 50% (EC_{50} values) were 15 μM for the T cells and 35 μM for the B cells. However, the cytotoxicity of deoxyguanosine could be partially ameliorated in the 8402 cells by the addition of 50 μM deoxycytidine to the culture
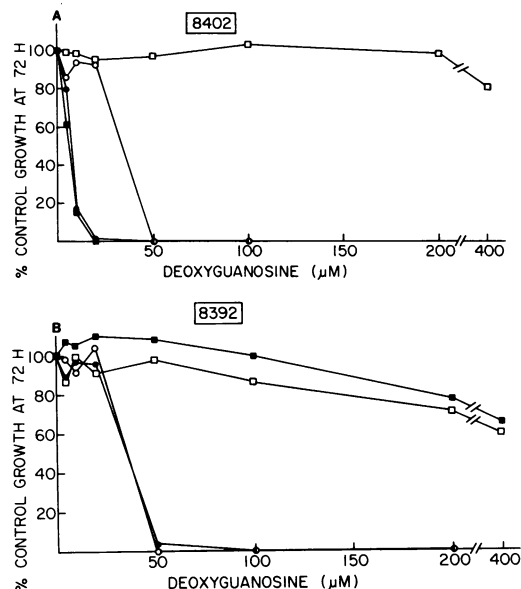

Figure 1. Comparison of the growth sensitivities of 8402 and 8392 cells to deoxyguanosine. The growth inhibitory effects of increasing deoxyguanosine concentrations were examined for the 8402 T (*A*) and 8392 (*B*) cell lines as described in Methods. Each cell line was tested in the absence (○) or presence of either 50 μM deoxycytidine (⊙), 50 μM adenine (■), or both 50 μM deoxycytidine and 50 μM adenine (□). Each datum is plotted as a percentage of control growth lacking deoxyguanosine. This experiment has been repeated two other times with similar results.

medium, whereas deoxycytidine had virtually no effect on the cytotoxicity of deoxyguanosine in B cells (Fig. 1). Addition of 50 μM adenine to the growth medium that contained deoxycytidine further protected 8402 cells from the cytotoxic effects of deoxyguanosine (Fig. 1). Conversely, 50 μM adenine alone in the growth medium substantially protected 8392 cells, but not 8402 cells, from deoxyguanosine cytotoxicity. Neither adenine nor deoxycytidine in the absence of deoxyguanosine had any growth inhibitory on these human cell lines. These results suggested that the primary mechanisms of deoxyguanosine cytotoxicity in 8402 and 8392 cells were markedly different.

Therefore, the abilities of the 8402 and 8392 cells to metabolize deoxyguanosine to the nucleotide level were compared. As shown in Fig. 2 *A*, only the 8402 T cells could convert deoxyguanosine to dGTP. High concentrations of exogenous deoxyguanosine increased intracellular dGTP levels several hundred-fold in 8402 cells, whereas no elevation in dGTP pools was observed in the B cells (Fig. 2). Conversely, deoxyguanosine caused a massive increase in cellular GTP concentrations in the B cells (Fig. 2 *B*) with a concomitant depletion of intracellular ATP pools (data not shown). GTP levels were also increased in T cells at low exogenous deoxyguanosine concentrations, but somewhat surprisingly not at higher deoxyguanosine concentrations. The selective conversion of deoxyguanosine to dGTP in 8402 cells could be abolished by exogenous deoxycytidine (Fig. 2 *C*). Deoxyguanosine ac-
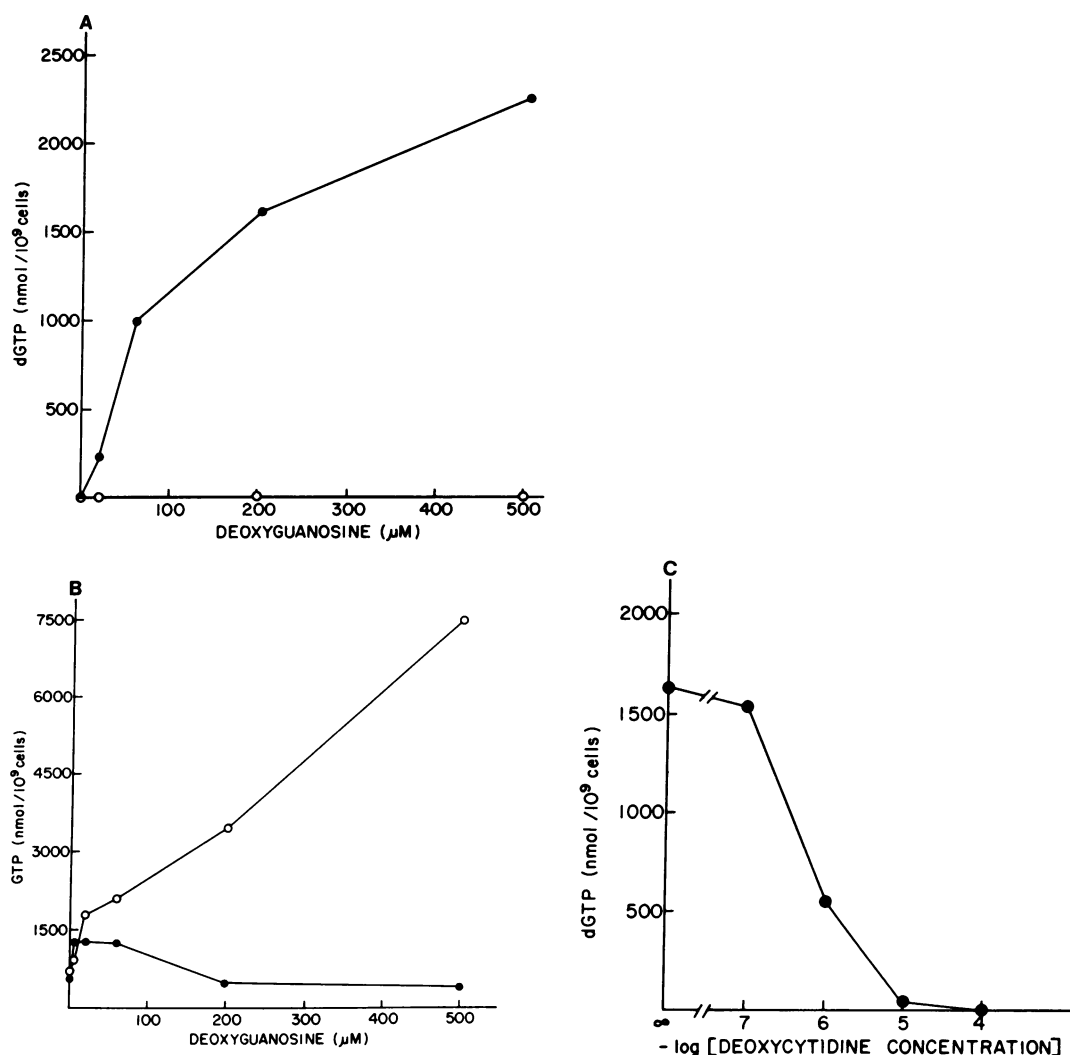
Figure 2. Deoxyguanosine metabolism in 8402 and 8392 cells. 8402
T (●) and 8392 B (○) cells were incubated with various exogenous
deoxyguanosine concentrations for 4 h and the intracellular dGTP (A
and C) and GTP (B) quantitated by high performance liquid chro-
matography as described in Methods. The effect of varying deoxycyti-
dine concentrations on the ability of intact 8402 cells to convert 0.2
mM deoxyguanosine into dGTP is depicted in C. The results de-
picted are those of a single experiment that has been repeated one
other time with similar results.

cumulation to dGTP by 8402 cells was inhibited ~65% by 1
μM deoxycytidine. Deoxycytidine did not interfere with the
conversion of deoxyguanosine to GTP in B cells (data not
shown). The prevention of deoxyguanosine accumulation to
the triphosphate and the protection of deoxyguanosine cyto-
toxicity by deoxycytidine indicated that deoxycytidine-deoxy-
guanosine kinase initiated the metabolism of deoxyguanosine
in the T cell line.

The above data suggested that deoxyguanosine analogues
which are resistant to cleavage by PNP might be selectively
metabolized and cytotoxic toward the T cell line. The prototype
of such an analogue is arabinosylguanine, which is not a
substrate for PNP (14). The sensitivities of the 8402 and 8392
cell lines to the cytotoxic effects of arabinosylguanine are

compared in Fig. 3. Whereas 8402 and 8392 cells exhibited
relatively similar growth sensitivity toward deoxyguanosine,
arabinosylguanine was much more selectively cytotoxic toward
the 8402 cells. The EC_{50} values obtained for arabinosylguanine
were 0.3 μM for 8402 T cells and 125 μM for 8392 B cells
(Fig. 3). The selective cytotoxicity of arabinosylguanine for the
8402 cell line could be eliminated completely by 50 μM
deoxycytidine (Fig. 3). We also compared the abilities of both
cell lines to accumulate arabinosylGTP. From 100 μM arabi-
nosylguanine in the culture medium, 8402 cells accumulated
76 nmol arabinosylGTP/10^9 cells after 4 h, while 8392 cells
accumulated <6 nmol arabinosylGTP/10^9 cells. ArabinosylGTP
accumulation was accompanied by a fourfold increase in
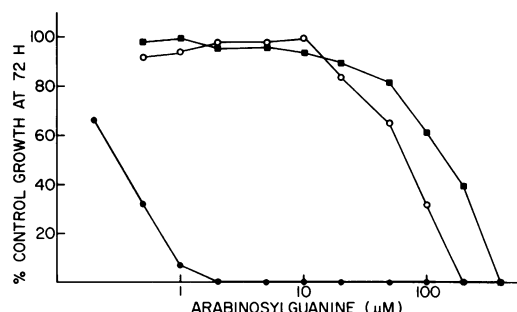cellular dATP levels, while other nucleotide pools remained

Figure 3. Comparison of the growth inhibitory effects of arabinosyl-guanine on 8402 and 8392 cells. The growth inhibitory and cytotoxic effects of arabinosylguanine were examined with 8402 T cells in the absence (●) or presence (○) of 50 μM deoxycytidine and with 8392 B cells (■). The results of this experiment were typical of three others performed under identical conditions.

unperturbed (data not shown). This selective metabolism of exogenous arabinosylguanine to arabinosylGTP by 8402 cells appears to be mediated by deoxycytidine kinase, since deoxy-cytidine could abolish this accumulation of arabinosylGTP. Moreover, somatic cell mutants of a murine T lymphoma (S49) that is resistant to arabinosylguanine toxicity have been generated, all of which are defective in their ability to accu-mulate arabinosylGTP via the deoxycytidine kinase (personal observations).

Discussion

Traditional chemotherapeutic regimens toward lymphocytic malignancies have been directed toward parameters which reflect the relatively high rates of metabolism or replication of the tumor cell population. Such agents, however, have relatively little specificity, and effect every normal cell that is undergoing DNA synthesis or repair. An ideal agent for the treatment of lymphoproliferative diseases would be a drug that is selective for the malignant cells. The discoveries of the association of deficiencies in adenosine deaminase (ADA) or purine nucleoside phosphorylase with immunodeficiency diseases in humans (1, 18) have stimulated considerable interest in the design of selective agents for the treatment of lymphoproliferative diseases or for immunosuppression. Initial approaches to the design of specific chemotherapies have focused on pharmacologic inhi-bition of ADA activity. Administration of deoxycoformycin (19), a potent inhibitor of ADA activity, to patients with acute lymphoblastic leukemia, causes a marked reduction in tumor cell burden (20–22). However, deoxycoformycin also eliminates normal T and B cell populations, which is not unexpected since ADA deficiency in humans affects both cellular and humoral immunity, and causes serious cytotoxicities to other organs including the kidneys, lungs, eyes, and central nervous system (21, 22).

In contrast to the combined T and B cell immunodeficiency that accompanies ADA deficiency, PNP deficiency causes a specific T cell abnormality with apparently normal B cell function. The results of cell culture studies (2, 5–9, 14, 16)

and the discovery of elevated dGTP in erythrocytes of PNP-deficient patients (3) have implicated deoxyguanosine as the cytotoxic substrate of PNP. Unfortunately, pharmacologic inhibitors of PNP such as formycin B (23) or 8-aminoguanosine (24) have limited clinical applicability due to their relatively low affinity for the enzyme. Conversely, in vivo administration of deoxyguanosine to leukemic patients in the absence of inhibitor is ineffective, since deoxyguanosine is rapidly cleaved to guanine by erythrocytic PNP (25). An alternative approach to the eradication of T cell malignancies or to the modulation of T cell functions would be the utilization of cytotoxic deoxyguanosine analogues that are resistant to degradation by PNP, such as arabinosylguanine (14). As a potential chemo-therapeutic agent, arabinosylguanine, a cytotoxic substrate of the deoxycytidine-deoxyguanosine kinase activity, should be more efficacious than an enzyme inhibitor such as deoxyco-formycin, which is dependent upon the accumulation and subsequent metabolism of deoxyadenosine, the cytotoxic sub-strate of ADA. Moreover, deoxyadenosine is generated from the degradation of DNA and is, thus, a product of deoxyaden-osine-deoxycoformycin toxicity per se.

We have compared the cytotoxicity and metabolism of both deoxyguanosine and arabinosylguanine, a deoxyguanosine analogue that is resistant to PNP cleavage, in syngeneic T and B cells; i.e., cells established from the same patient. Thus, differences in drug metabolism should theoretically reflect differential gene expression rather than genetic differences in biochemical targets. Although both 8402 and 8392 cells were sensitive to deoxyguanosine, the mechanisms of cytotoxicity were different. The primary mechanism of deoxyguanosine cytotoxicity in 8402 cells, i.e., the component protected by deoxycytidine, was mediated by direct phosphorylation that was initiated by deoxycytidine-deoxyguanosine kinase. A sec-ondary mechanism of deoxyguanosine cytotoxicity in these T cells and the sole mechanism involved in the killing of 8392 B cells, reversible by adenine, involves the conversion of deoxyguanosine to guanylate nucleotides via PNP and hypoxanthineguanine phosphoribosyltransferase. Guanylate nucleotides cannot be converted to inosine monophosphate in mammalian cells and their inhibitory effects on the early part of the purine biosynthetic pathway (5) deplete cells of adenylate nucleotide pools.

The unique ability of T cells to accumulate dGTP from exogenous deoxyguanosine suggested that deoxyguanosine an-alogues that are resistant to phosphorolysis by PNP might be cytotoxic exclusively to cells of thymic origin. We, therefore, compared the cytotoxicities of arabinosylguanine, a non-PNP substrate (14), on the syngeneic 8402 T and 8392 B cells. Whereas the T cells were only 2.3-fold more sensitive than B cells to deoxyguanosine, they were >400-fold more sensitive to arabinosylguanine toxicity. This large difference between the syngeneic cells toward arabinosylguanine could be attributed to the unique ability of 8402 cells to accumulate arabinosylGTP. Arabinosylguanine was not metabolized in the 8392 cells.

Although PNP deficiency is an inborn error of metabolism afflicting <20 patients, the elucidation of the metabolic basis

of the specific lymphotoxicity has provided biochemical rationale for the design of a T cell specific agent. T cells have the unique ability to accumulate the triphosphate of deoxyguanosine and some of its analogues, such as arabinosylguanine. In the presence of deoxycytidine, which renders 8402 cells incapable of this triphosphate accumulation, the syngeneic 8402 and 8392 cells were equally sensitive to both deoxyguanosine (EC values equal 35 μM) and arabinosylguanine (EC values equal 100 μM). Despite the exquisite sensitivity of 8402 and other T cells (14) to arabinosylguanine, the clinical usefulness of arabinosylguanine is probably limited by its low solubility and high frequencies of cellular resistance (data not shown). We have also tested three other deoxyguanosine analogues, which are all 6-thioguanine formycin C-nucleosides, in our cell culture system. None were growth inhibitory to 8402 or 8392 cells. Nevertheless, the design of deoxyguanosine or other deoxyribonucleoside analogues that are selectively accumulated to the nucleotide level by T cells offers a rational approach for the treatment of T cell malignancies or for the treatment of other T cell disorders, such as autoimmune abnormalities or graft vs. host disease.

Acknowledgments

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