A Biochemical Genetic Study of the Role of Specific Nucleoside Kinases in Deoxyadenosine Phosphorylation by Cultured Human Cells*  

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Deoxyadenosine is a toxic substrate of adenosine deaminase and accumulates as deoxyATP in cells of patients deficient in that enzyme. However, it is unclear which of the deoxyadenosine phosphorylating enzymatic activities is responsible for this accumulation of deoxyATP in humans. From a human lymphoblastoid cell line, WI-L2, mutants deficient in various nucleoside kinase enzymes have been isolated. A line of cells lacking deoxycytidine kinase had less than 10% of the deoxyadenosine phosphorylating activity of the wild type cells when measured in cell free extracts. However, in growth rate experiments, this cell line was as sensitive as the wild type cell line to the toxic effects of deoxyadenosine. Furthermore, these mutant cells could accumulate deoxyATP from exogenous deoxyadenosine as effectively as the wild type cells.

A second line of cells (107A), deficient in adenosine kinase activity, had virtually wild type levels of deoxyadenosine phosphorylating activity when measured in extracts. However, the cells of this line were 3-fold less sensitive than wild type cells to deoxyadenosine and accumulated far less intracellular deoxyATP from exogenous deoxyadenosine. A cell line lacking both adenosine kinase and deoxycytidine kinase activities exhibited 3- to 5-fold greater resistance to the growth inhibitory and toxic effects of deoxyadenosine than did the adenosine kinase deficient parent and did not accumulate detectable deoxyATP from exogenous deoxyadenosine.

Although deoxyadenosine phosphorylating activity in cell extracts appears to be associated with the deoxycytidine kinase enzyme, it is clear that the physiologically important deoxyadenosine phosphorylating activity is associated with the adenosine kinase enzyme. Thus, the roles of specific nucleoside kinases in the metabolism of deoxyadenosine in human cells have been delineated by these biochemical genetic studies.

In 1972, Giblett and co-workers reported that the erythrocytes of a patient with severe combined immunodeficiency disease lacked adenosine deaminase (1). Subsequently among the population of immunodeficient patients, approximately 50 to 100 other individuals have been discovered who are lacking the erythrocytic adenosine deaminase activity (2). Both adenosine and deoxyadenosine serve as natural substrates for adenosine deaminase (3-5). It is likely that deoxyadenosine serves as a potentially toxic substrate in adenosine deaminase deficiency since extraordinarily high concentrations of deoxyadenosine nucleotides have been found in the erythrocytes and lymphocytes of adenosine deaminase-deficient, immunodeficient patients (6-9). Furthermore, after transfusion of a small quantity of irradiated erythrocytes containing normal adenosine deaminase to adenosine deaminase-deficient patients, deoxyATP levels are reduced (6). In at least some patients, this decrease in deoxyATP has been accompanied by transient restoration of immunocompetency (10). A major form of deoxyadenosine toxicity in cultured cells seems to be mediated by deoxyATP inhibition of the ribonucleotide reductase (11-14), the enzyme which reduces the 2'-hydroxy moieties of all four ribonucleoside diphosphates to generate the 2'-deoxyribonucleotides required for DNA synthesis.

Ullman et al. demonstrated that deoxyadenosine toxicity in mutant murine T-lymphocytic lymphoma (S49) cells requires transport across the plasma membrane and intracellular phosphorylation to deoxyATP (14). This characteristic of deoxyadenosine toxicity led to a study of the cellular nucleosides responsible for deoxyadenosine phosphorylation. It has been suggested that mammalian cells contain separate nucleoside kinases for adenosine and deoxyadenosine (15-19). Although most preparations of purified deoxycytidine kinase have been shown to phosphorylate deoxyadenosine (20, 21), in S49 cells the majority of the phosphorylation of deoxyadenosine and adenosine is carried out by the single enzyme, adenosine kinase (14). Mutant S49 cells deficient in adenosine kinase activity are 4-fold less sensitive than wild type cells to the growth-inhibitory effects of deoxyadenosine (14). An S49 cell line deficient in deoxycytidine kinase is as sensitive as the wild type cell line to deoxyadenosine and accumulates deoxyATP from deoxyadenosine, indicating that the major route of deoxyadenosine phosphorylation occurs through adenosine kinase (14). Furthermore, a mutant cell line deficient in both deoxycytidine kinase and adenosine kinase is even more resistant to the growth inhibitory and toxic properties of deoxyadenosine (14). This observation in S49 cells suggested that although in wild type cells the majority of the phosphorylation of deoxyadenosine is carried out by adenosine kinase, a low level of deoxyadenosine phosphorylation occurs via its deoxycytidine kinase.

Recently, Hershfield et al. have shown that a human B-lymphoblast (WI-L2) line deficient in adenosine kinase is less sensitive to deoxyadenosine than its wild type parent (22). The adenosine kinase-deficient cells accumulate less deoxyATP and are less susceptible to S-adenosylhomocys-
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tyidine kinase inactivation by deoxyadenosine than are wild type cells (22, 23).

From wild type and from the adenosine kinase-deficient WI-L2 cell lines, we have isolated mutants deficient in deoxy-
cytidine kinase. Extracts from the enzyme-deficient cell lines were examined for deoxyadenosine phosphorylating activities, and the sensitivities of the intact mutant cells to the growth inhibitory properties of exogenous deoxyadenosine were studied. These cultured human cells exhibit the same pattern of dependence on specific nucleoside kinases for the metabolism of deoxyadenosine as do the murine S49 cells.

MATERIALS AND METHODS

Radioactively labeled [8-\(^{14}\)C]deoxyadenosine (37.3 pmCi/mmol), [8-
\(^{14}\)C]deoxyadenosine (55 mCi/mmol), and [2-\(^{14}\)C]deoxycytidine (29.7 mCi/
millol) were all purchased from New England Nuclear Corp. The 9-
erthro-(2-hydroxy-3-nonyl)-adenine was obtained from Burroughs-Welcomes Corp. The sources of all other materials, chemicals, and reagents have been reported previously and were of the highest qualities commercially available (24, 25).

Cell Culture and Mutant Selection—The splenic-derived human B-cell line, WI-L2, was initially described by Levy et al. (26). The wild type and mutant WI-L2 cells were normally maintained in suspension in 75-cm\(^2\) Falcon T flasks in a humidified 10% CO\(_2\) incubator at 37°C. All cells were grown in Dulbecco's modified Eagles' medium containing 3.7 g/liter of sodium bicarbonate and 3 g/liter of b-glucose, and supplemented with horse serum that had been preincubated and heat-inactivated at 56°C for 30 min. Cultures were routinely grown by dilution with fresh medium every 2 or 3 days. The horse serum utilized in these experiments contained less than 0.02 mmol/min activity of adenosine deaminase/mg of protein. The adenosine kinase-deficient WI-L2 cell line (107A) was isolated by Hershfield et al. (27) by virtue of its resistance to the toxic adenine analog, 6-methylmercapto purine ribonucleoside. To isolate the deoxy- cytidine kinase-deficient lines from wild type and from 107A cells, 200 ml of exponentially growing cells at a density of 10\(^{6}\) cells/ml were exposed to ICR-191 at a concentration of 2 \(\mu\)g/ml. After a 16-h exposure to the mutagen, cells were sedimented by centrifugation, diluted into fresh medium, and allowed to grow nonselectively for 5 days. The cells were subsequently exposed to 10 \(\mu\)M arabinosylcytosine in suspension culture for 24 h and cloned in agarose containing arabinosylcytosine overlaying mouse embryo fibroblast feeder layers (28). The deoxycytidine kinase-deficient cell line (WI-L2 AK \(-\)araC-6) was isolated from wild type cells due to its resistance to 7 \(\mu\)M arabinosylcytosine. The deoxycytidine kinase-deficient cell line (WI-L2 AK \(-\)araC-6) derived from the adenosine kinase-deficient WI-L2 cells (107A) was isolated in agarose containing 6 \(\mu\)M arabinosylcytosine. Cloning efficiencies varied between 30 and 50% in multiple experiments, and the frequency of the arabinosylcytosine-resistant clones was less than 10\(^{-7}\).

Growth Experiments—The experiments to determine the ability of wild type and mutant WI-L2 cells to grow in the presence of various chemical agents were conducted in Costar multiwell (24 well) tissue culture plates as described previously for S49 cells (14). Small volumes (10 to 50 \(\mu\)l) of growth inhibitory agent were pipetted into each well after which 10\(^{5}\) exponentially growing cells were added at a density of 10\(^{6}\) cells/ml. After 72 h, the number of control cells (in the absence of added chemical agents) had typically increased 12- to 25-
fold (3.5 to 4.2 cell doublings). The initial cell densities were subtracted from the final cell densities, and the number of cells in the wells containing the growth inhibitory agents was plotted as the percentage of the number of cells in the control wells.

Enzyme Assays—Cell extracts were prepared in the following manner: 3 to 5 \(\times\) 10\(^{6}\) cells were harvested by centrifuga-
tion, washed with cold phosphate-buffered saline, and resuspended in a small volume of 20 mM Tris buffer, pH 7.4, 10% glycerol, 1 mM dithiothreitol, and 0.5% Nonidet 40. The cells were lysed by freezing and thawing three times. Extracts were centrifuged in the Beckman microfuge B at 10,000 \(\times\) g for 1 min, and the supernatants were sieved over a Sephadex G-25 column (0.5 x 20 cm), previously equilibrated with the lysis buffer. Adenosine kinase activity was measured by the method of Gudas et al. (29), and deoxyadenosine and deoxycytidine phosphorylating activities were measured by the method of Ullman et al. (14).

Deoxyadenosine Accumulation as Deoxyadenosine Triphosphate—The capacities of intact wild type and mutant WI-L2 cells to

![Fig. 1. Effect of deoxyadenosine on the growth rates of wild type and mutant WI-L2 cells. Different amounts of deoxyadenosine were added to exponentially growing wild type cells ( ), adenosine kinase-deficient cells ( ), deoxycytidine kinase-deficient cells ( ), and cells deficient in both adenosine kinase-deoxycytidine kinase ( ), in the presence of 10 \(\mu\)M EHNA. Cell growth is plotted as a percentage of cell growth after 72 h in the absence of deoxyadenosine. These data are those of a typical experiment which has been repeated at least eight times with similar results.](image)
exclusively by adenosine kinase. Adenosine at a (non-growth-inhibitory) concentration of 20 to 25 μM increases the EC50 values for deoxyadenosine-EHNA by no more than 2-fold in all four cell lines, i.e. wild type, adenosine kinase-deficient, deoxycytidine kinase-deficient, and deoxycytidine kinase-deoxycytidine kinase-deficient cells. The effects of exogenous deoxycytidine on the growth of wild type and the mutant cells are shown in Fig. 2. Deoxycytidine will compete with deoxyadenosine and 10 μM EHNA in the presence of deoxycytidine, but not adenosine kinase-deficient cells by 4-fold, but does not significantly alter the EC50 value for the deoxycytidine kinase-deficient mutants. As expected, in the presence of deoxycytidine, the EC50 value for deoxyadenosine-EHNA in the adenosine kinase-deficient mutant is comparable to that of the double mutant without added deoxycytidine since the presence of deoxycytidine effectively eliminates phosphorylation of deoxyadenosine by deoxycytidine kinase.

**Enzyme Studies**—The capacities of extracts of all four human WI-L2 cell lines to phosphorylate adenosine, deoxy-

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**Fig. 2.** Effect of deoxycytidine on deoxyadenosine mediated growth inhibition of wild type and mutant WI-L2 cells. Wild type and mutant WI-L2 cells were incubated with varying concentrations of deoxyadenosine and 10 μM EHNA in the presence (---) and absence (-----) of 50 μM deoxycytidine. A, wild type (••••) and deoxycytidine kinase-deficient (○○○○) cells. B, adenosine kinase-deficient (□□□□) and deoxycytidine kinase-deoxycytidine kinase-deficient (■■■■) cells. This is a representative experiment which has been repeated three times with similar results.

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**Fig. 3.** Levels of nucleoside phosphorylating activities in cell-free extracts of wild type and mutant WI-L2 cells. The capacities of extracts of wild type (••••), adenosine kinase-deficient (○○○○), deoxycytidine kinase-deficient (□□□□), and adenosine kinase-deoxycytidine kinase-deficient (■■■■) cells to phosphorylate adenosine (Panel A), deoxyadenosine (Panel B), and deoxycytidine (Panel C) are shown. The procedures are those described under "Materials and Methods" using as the phosphate acceptor the substrate nucleoside at a concentration of 50 μM. These results have been reproduced at least three other times.

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**Fig. 4.** Accumulation of deoxyATP in WI-L2 wild type and mutant cell lines. Fifty-milliliter cultures of WI-L2 cells (0.8 to 1.1 x 10⁶/ml) in Dulbecco modified Eagles’ Medium + 10% horse serum were incubated with 10 μM EHNA. Thirty minutes later, deoxyadenosine at either 50 μM or 500 μM with and without equimolar concentrations of adenosine or deoxycytidine was added and the culture was incubated at 37°C for 4 h. The cell extracts were prepared and deoxyATP levels were high by performance liquid chromatography as described under "Materials and Methods." The unperturbed control levels of deoxyATP in wild type cells varied in different experiments from 0.02 to 0.04 nmol of deoxyATP/10⁶ cells. In the mutant cell lines, the control levels (without added deoxyadenosine) varied in different experiments from 0.01 to 0.025 nmol deoxyATP/10⁶ cells. The depicted values of per cent change over control represent the mean of three experiments for the wild type and two experiments for the mutant cells. Compared to the control values, the levels of deoxyATP did not vary more than 10% between experiments. The open bars greater than zero represent values of deoxyATP above the addition of deoxyadenosine (dAdo) alone. The solid bars and the cross-hatched bars represent values of deoxyATP after the addition of deoxyadenosine and equimolar adenosine (Ado) or deoxycytidine (dCyd), respectively.
Rates of Deoxynucleoside Uptake—The capacity of intact WI-L2 cells preincubated with 10 μM EHNA to accumulate intracellular deoxycytidinosine nucleotides after exposure to exogenous deoxyadenosine is shown in Fig. 4. It is clear that under those conditions at least 90% of the accumulation of deoxyadenosine nucleotides from deoxyadenosine at a concentration of either 50 μM or 500 μM is dependent upon an intact adenosine kinase activity. Therefore, the capacity of intact cells to phosphorylate deoxyadenosine is dependent upon the adenosine kinase enzyme, whereas the capacity of extracts of WI-L2 cells to phosphorylate deoxyadenosine is associated with the deoxycytidine kinase enzyme.

**DISCUSSION**

The issue of which human nucleoside kinase is primarily responsible for deoxyadenosine phosphorylation has engendered considerable debate (22, 23, 32–35). The controversy stems from the fact that the majority of the deoxyadenosine phosphorylating activity in S49 cell extracts is associated with adenosine kinase (14), whereas in extracts of human F-lymphoblasts, the deoxyadenosine phosphorylating activity seems to be associated with deoxycytidine kinase (18, 19, 22, 23). Recent data from Carston’s laboratory (22) have suggested that intact cells may phosphorylate deoxyadenosine via both adenosine kinase and deoxycytidine kinase (35). We have attempted to resolve this debate by isolating mutants of cultured human (WI-L2) cells deficient in adenosine kinase, deoxycytidine kinase, or both enzymes. These mutant cells have been studied by comparing their abilities to grow in the presence of exogenous deoxyadenosine and measuring their deoxyadenosine phosphorylating capacities in intact cells and cell extracts. It is clear that in extracts of WI-L2 cells the majority of the deoxyadenosine phosphorylating activity is associated with the deoxycytidine kinase enzyme. However, the deoxycytidine kinase-deficient cell line is normal sensitive to deoxyadenosine and can efficiently accumulate deoxyATP from deoxyadenosine. Resistance to deoxyadenosine in growth rate experiments is conferred by a mutation which eliminates adenosine kinase activity and which concomitantly reduces the ability of the cells to accumulate intracellular deoxyATP from extracellular deoxyadenosine. However, cell-free extracts of the adenosine kinase-deficient cells can phosphorylate deoxyadenosine as effectively as wild type cell extracts. This suggests that the measurements of deoxyadenosine phosphorylating activity in extracts of human cell lines may lead to erroneous conclusions as to the identity of the physiologically and pathologically important deoxyadenosine phosphorylating enzyme. The known ability of dCTP and dGTP to inhibit phosphorylation of deoxyadenosine by deoxycytidine kinase (36) may affect the enzyme function in the intact cell.

The apparent discrepancy between studies on deoxyadenosine metabolism in cell-free extracts and in intact cultured cells is not well understood. The two deoxyadenosine phosphorylating activities which can phosphorylate deoxyadenosine have different Km values for deoxyadenosine. The Km value for deoxyadenosine of the adenosine kinase is approximately 500 to 540 μM (22, 23), whereas that of deoxycytidine kinase is approximately 120 μM for deoxyadenosine (22, 23). Furthermore, the two enzymatic activities have different magnesium and pH optima (14, 18, 22). It is not known what role, if any, these factors play in this discrepancy.

Hershel and Kredich (22, 23) have proposed that deoxyadenosine may be lymphotoxic via its irreversible inactivation of the enzyme S-adenosyl-L-homocysteine hydrolase (37). This inactivation would raise intracellular levels of S-adenosylhomocysteine, a potent inhibitor of S-adenosylmethionine-dependent methylation reactions essential for cellular homeostasis (38). Hershel et al. (22, 23) have proposed that the adenosine kinase-deficient (107A) cell line may be resistant to deoxyadenosine by virtue of elevated endogenous adenosine, which can compete with deoxyadenosine for binding to the hydrolase. Intact 107A cells also accumulate less deoxyATP from deoxyadenosine (Ref. 22 and Fig. 4) than do wild type cells, and adenosine decreases the accumulation of deoxyATP in wild type cells. Thus, it appears that phosphorylation to deoxyATP may play a significant role in the lymphotocicity of deoxyadenosine in human B-lymphoblasts. Addition of deoxycytidine to the culture medium noticeably diminishes the sensitivity of wild type and adenosine kinase-deficient cells to deoxyadenosine (Fig. 2, A and B), consistent with a concomitance of a deoxyATP-mediated inhibition of cytidine diphosphate reduction (11, 14, 39). The effect of added deoxycytidine in wild type cells clearly is not due to its competing with deoxyadenosine for phosphorylation by deoxycytidine kinase since even the complete deficiency of that enzyme does not convey resistance to deoxyadenosine. The double mutant, deficient in both deoxycytidine kinase and adenosine kinase, which has the least amount of deoxyadenosine phosphorylating capacity measured in extracts, is the least sensitive to the growth inhibitory and toxic properties of deoxyadenosine. These studies of deoxyATP accumulation and growth rates in human cell mutants exposed to deoxyadenosine are consistent with a role for ribonucleotide reductase in deoxyadenosine toxicity in WI-L2 cells.

The role of deoxyadenosine phosphorylating activities in human cell lines is potentially important in the design of specific chemotherapeutic agents which might be used to modulate lymphoproliferative disorders in humans (40).

**REFERENCES**

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