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SUBSTRATE SPECIFICITY OF HUMAN DEOXYCYTIDINE KINASE TOWARD ANTIVIRAL 2′,3′-DIDEOXYNUCLEOSIDE ANALOGS

BORYS KIERDASZUK,*† CHRISTINA BOHMAN,* BUDDY ULLMAN‡§ and STAFFAN ERIKSSON*

*Medical Nobel Institute, Department of Biochemistry I, Karolinska Institute, S-10401 Stockholm, Sweden; †Department of Biophysics, Institute of Experimental Physics, University of Warsaw, 02-089 Warsaw, Poland; and ‡ Department of Biochemistry, The Oregon Health Sciences University, Portland, OR 97201, U.S.A.

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Abstract—Deoxycytidine (dCyd) kinase has been purified to homogeneity from human leukemic spleen, and the capacity of the enzyme to phosphorylate 2′,3′-dideoxynucleoside (ddN) analogs that are clinically effective inhibitors of human immunodeficiency virus (HIV) replication was evaluated. Cytosine-containing ddN analogs, such as 2′,3′-dideoxycytidine, 2′,3′-dideoxy-2′,3′-dehydrocytidine, and cytallene, were efficiently phosphorylated by dCyd kinase, while no phosphorylation of purine-containing ddN analogs was detected. dCyd kinase was completely inactive toward 2′,3′-dideoxyadenosine (ddAdo), 2′,3′-dideoxyinosine, 2′,3′-dideoxyguanosine, and adenallene, although it was capable of phosphorylating both 2′-deoxyadenosine (dAdo) and 2′-deoxyguanosine (dGuo). The abilities of wild type and mutant human T lymphoblastoid CEM cells to accumulate ddAdo *in situ* and *in vitro* were also ascertained. Comparison of the abilities of intact wild type CEM cells and derivatives deficient in nucleoside transport, dCyd kinase, and/or adenosine (Ado) kinase to accumulate [^{3}H]ddAdo-derived radioactivity revealed no significant differences among the wild type and mutant strains. However, ddAdo phosphorylating activity was decreased in extracts from Ado kinase-deficient cells but not in lysates prepared from cells genetically deficient in dCyd kinase activity. In comparative growth rate experiments, wild type, nucleoside transport-deficient, and dCyd kinase-deficient CEM cells were equally sensitive to ddAdo toxicity, while, interestingly, a deficiency in Ado kinase correlated with a 5-fold decreased growth sensitivity to the purine ddN. Insertion of an adenine phosphoribosyltransferase deficiency into the CEM cell lines did not influence ddAdo toxicity or incorporation rate. These results imply that Ado kinase may be an important factor in ddAdo phosphorylation by CEM cells. Furthermore, these studies demonstrate that cytosine- and purine-containing ddNs are transported and activated by independent pathways and, therefore, have important implications for anti-HIV therapy in that pyrimidine and purine ddNs might be used in combination for the treatment of acquired immunodeficiency syndrome.

2′,3′-Dideoxynucleosides (ddNs‖) are potent inhibitors of human immunodeficiency virus (HIV) replication and have been exploited clinically to treat patients with acquired immunodeficiency syndrome (AIDS). The antiviral effects of these ddNs require their phosphorylation to the corresponding ddN triphosphate. A biochemically rational strategy for the exploitation of ddNs in the treatment of AIDS will depend on a fundamental understanding of the metabolic pathways by which these compounds are converted to their active metabolites. The best available data suggest that all the phosphorylation steps for ddNs are catalyzed by the metabolic machinery of the cell, most notably the cellular nucleoside kinases [1–8]. Evidence obtained with partially purified enzyme preparations and with mutant cell lines has suggested that the cellular deoxycytidine (dCyd) kinase is primarily responsible for the phosphorylation of a spectrum of purine and pyrimidine ddNs [2, 4–11]. For instance, there is convincing kinetic evidence that dCyd kinase is required for the biological activity of 2′,3′-dideoxycytidine (ddCyd), although these studies with the partially purified enzyme indicated that ddCyd is a relatively poor substrate [4, 5, 10, 11]. Moreover, mammalian cell lines genetically deficient in dCyd kinase are resistant to the cytotoxic and antiviral effects of ddCyd and are incapable of converting radiolabeled ddCyd to the nucleotide level [2, 12, 13].

Whether dCyd kinase catalyzes the phosphorylation of the purine ddN analog 2′,3′-dideoxyadenosine (ddAdo) is less clear. Johnson *et*

§ Corresponding author. Tel. (053) 494-8437; FAX (503) 494-8393).

‖ Abbreviations: ddN, 2′,3′-dideoxynucleoside; ddAdo, 2′,3′-dideoxyadenosine; ddCyd, 2′,3′-dideoxycytidine; ddGuo, 2′,3′-dideoxyguanosine; ddIno, 2′,3′-dideoxyinosine; N^1-(4′-hydroxy-1′,2′-butadienyl)cytosine; adenallene, N^9-(4′-hydroxy-1′,2′-butadienyl)adenine; D4C, 2′,3′-dideoxy-2′,3′.dehydrocytidine; D4T, 2′,3′-dideoxy-2′,3′-dehydrothymidine; araCyt, arabinosylcytosine; Ado, adenosine; dCyd, deoxycytidine; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; DAP, 2,6-diaminopurine; NBMPR, 4-nitrobenzylthioinosine; DPA, dipyridamole; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; APRTase, adenine phosphoribosyltransferase; HGPRTase, hypoxanthine-guanine phosphoribosyltransferase; HIV, human immunodeficiency virus; and AIDS, acquired immunodeficiency syndrome.

al. [6, 8] have indicated that the partially purified dCyd kinase preparations from human thymus and CEM T lymphoblastoid cells, as well as the partially purified CEM cell adenosine (Ado) kinase, could convert ddAdo to the nucleotide level. Employing intact CEM cells, Carson *et al.* [7] showed that a dCyd kinase-deficient mutant can accumulate ddATP from exogenous ddAdo as effectively as wild type parental cells, and that the antiviral efficacy of ddAdo is the same in both wild type and mutant cells genetically deficient in both dCyd and Ado kinase activities. In contrast, Johnson *et al.* [8] indicated that dCyd kinase-deficient CEM cells, as well as an Ado kinase-deficient mutant, are indeed less capable than wild type cells of accumulating ddATP from exogenous ddAdo. Furthermore, Sarup *et al.* [9] reported that a 2000-fold purified preparation of dCyd kinase from leukemic lymphoblasts phosphorylated ddAdo and 2′,3′-dideoxyguanosine (ddGuo), as well as ddCyd. However, the relative efficiency of the purine ddNs as substrates for this dCyd kinase preparation was approximately 1000-fold lower than that of the corresponding 2-deoxynucleosides, 2′-deoxyadenosine (dAdo) and 2′-deoxyguanosine (dGuo), and 100-fold lower than that of ddCyd.

To investigate the substrate specificity of a dCyd kinase enzyme in the absence of other cellular components, Bohman and Eriksson [14] have purified dCyd kinase from human leukemic spleen 6000-fold to apparent homogeneity. This has permitted an assessment of the ability of the purified enzyme to phosphorylate a variety of purine and pyrimidine ddN analogs in the absence of contaminating enzyme activities. Among the ddN analogs evaluated as potential substrates of dCyd kinase were ddCyd, ddAdo, 2′,3′-dideoxyinosine (ddIno), ddGuo, and two acyclic nucleoside analogs, cytallene and adenallene, and two olefinic ddNs, 2′,3′-dideoxy-2′,3′-dehydrocytidine (D4C) and 2′,3′-dideoxy-2′,3′-dehydrothymidine (D4T), all of which are known to be active against HIV [11, 15–17]. A preliminary communication describing part of this work has been published recently [18]. In addition, the roles of the nucleoside transport system and various nucleoside kinase activities in modulating ddAdo toxicity and accumulation of ddAdo-derived metabolites have been analyzed *in situ* and *in vitro* using the human CEM T lymphoblastoid cell line, a permissive host for HIV infection [7, 19].

MATERIALS AND METHODS

Materials. 2′,3′-[5,6-^{3}H]ddCyd (10 Ci/mmol) and 2′,3′-[8-^{3}H]dideoxyguanosine (3 Ci/mmol) were purchased from Moravek Biochemicals (La Brea, CA). 2′,3′-[2′,3′-^{3}H]ddAdo (30 Ci/mmol) was a gift from Dr. David Johns of the National Cancer Institute (NCI) (Bethesda, MD). [5-^{3}H]dCyd (25 Ci/mmol), [G-^{3}H]dAdo (20 Ci/mmol), and [γ-^{32}P]ATP (triethylammonium salt) (3000 Ci/mmol) were obtained from the Amersham Corp. (Arlington Heights, IL). N^1-(4′-Hydroxy-1′,2′-butadienyl)cytosine (cytallene) and N^9-(4′-hydroxy-1′,2′-butadienyl)adenine (adenallene) were provided by Dr. Samuel Broder, also of the NCI, and D4C and D4T were donations from Dr. Jan Balzarini of The Rega Institute (Leuven, Belgium). ddCyd, ddAdo, and ddGuo were bought from Pharmacia Inc. (Piscataway, NJ). All other chemicals, materials, and reagents were of the highest quality commercially available.

Purification of dideoxynucleosides. Dideoxynucleosides were purified prior to use by HPLC on a nucleosil C_{18} column (250 × 46 mm, particle size 5 μm) purchased from Scandinaviska Genetec (Sweden) using an LKB 2150 HPLC pump possessing a Rheodyne 7125 injector and two Shimadzu SPD-2A detectors. ddNs were eluted isocratically from the reversed phase column as follows: 5% methanol for dCyd and ddCyd, 10% methanol for ddGuo, and 15% methanol for dAdo and ddAdo.

Enzyme preparations. Cytoplasmic dCyd kinase was purified from human leukemic spleen cells as described previously by Bohman and Eriksson [14]. This purified preparation appeared homogeneous by sodium dodecyl sulfate (SDS) gel electrophoresis and possessed a specific activity 6000-fold greater than that of the crude extract, i.e. 200 nmol of dCMP formed per min per mg of protein. To measure dCyd kinase activities in CEM cells, crude cell extracts were prepared essentially as described by Johnson *et al.* [6]. Briefly, 5×10^7, wild type or mutant, CEM cells were lysed in a Dounce homogenizer with a loose fitting pestle in 0.5 mL of a buffer consisting of 50 mM Tris–HCl, pH 7.5, 1.0 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10% glycerol. The cell homogenates were centrifuged at 12,000 *g* for 10 min in an Eppendorf centrifuge, and the soluble fractions employed for the dCyd kinase assays. Protein in the extracts was determined as described by Bradford [20].

dCyd kinase activity was measured routinely by a radiometric assay at 37°. The pure human splenic enzyme was assayed in 0.2 mL of a mixture containing 40 mM Tris–HCl, pH 7.6, 1 mM ATP, 1 mM $MgCl_2$, 100 mM KCl, 0.1 mg bovine serum albumin, an appropriate concentration of the radiolabeled nucleoside substrate as indicated, and approximately 50 ng of purified human dCyd kinase. Nucleoside phosphorylation assays using extracts prepared from CEM cells were performed using similar conditions but with a 200 μM concentration of nucleoside, 1 mM dithiothreitol, and 5 mM ATP:$MgCl_2$, as well as 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an inhibitor of adenosine deaminase [21], to prevent deamination of dCyd kinase substrates that are also substrates of adenosine deaminase. The reactions were initiated by the addition of the purified enzyme and were terminated by the removal of 10- to 50-μL aliquots from the reaction mixture and spotting the liquid onto Whatman DE81 filter discs at 0, 10, 20, and 30 min. The filter discs were pooled, washed three times with 2 L of 5 mM ammonium formate, and dried, and the product was eluted with 0.5 mL of KCl:HCl (0.2 M:0.1 M) and quantitated by liquid scintillation spectrometry as described [14]. The initial reaction velocities and kinetic parameters were determined using linear regression analysis.

The ATP transfer assay was performed with 100 μM [^{32}P]ATP (0.7 mCi/mmol), 49 mM Tris–HCl, pH 7.6, 0.5 mM $MgCl_2$, 100 mM KCl, 0.5 mg/

mL bovine serum albumin, 40–200 ng of dCyd kinase, and 100 μM nucleoside in a total volume of 50 μL. The reaction was terminated after 60 min by boiling for 1 min, the mixture was centrifuged in an Eppendorf microfuge for 5 min at 12,000 *g*, and 5 μL of the supernatant was applied to a PEI-cellulose F (Merck) thin-layer plate. Chromatography was performed for 2 hr using 99% isobutyric acid : 35% NH_4OH : H_2O (66 : 1 : 33, by vol.) as the mobile phase. In this thin-layer system, nucleoside monophosphates migrated with an R_f value between 0.5 and 0.7. The products of the kinase reaction were detected by autoradiography, and the spots were excised and eluted with 0.5 mL of KCl : HCl (0.2 M : 0.1 M), and quantitated by liquid scintillation.

High performance liquid chromatography. HPLC was employed to detect and identify products of the deoxynucleoside phosphorylation assay. Samples for analysis were prepared as follows. Nucleoside phosphorylation assays were performed at 37° as described above. After 1 hr, ice-cold methanol was added to quench the reaction to a concentration of 60%. Samples were incubated overnight at −20°, after which the precipitated proteins were removed by centrifugation at 12,000 *g* for 20 min. Supernatants were evaporated to dryness in a Buchler Vortex Evaporator and dissolved in 10 mM ammonium phosphate, pH 3.8, containing 2% methanol (buffer A). The analyses of the products of the kinase assays were performed by HPLC on a Whatman Partisil-10-SAX column (250 × 4.6 mm) equilibrated with buffer A and eluted first with buffer A for 10 min, followed by a linear gradient to 7% methanol and 0.5 M ammonium phosphate, pH 3.8, for the next 15 min as described by Spyrou and Reichard [22]. Nucleosides and nucleotides were detected and quantitated by their absorbances at 254 and 280 nm and by their coelution with known standards. Fractions (1 mL) from the anion exchange column were collected and their radioactive content was quantitated by liquid scintillation.

Cell lines and growth conditions. The origin and lymphocytic characteristics of the human T lymphoblastoid CEM cell-line have been described in detail [23]. The isolation and characterization of the CEM mutant cell lines genetically deficient in nucleoside transport (AraC-8C), Ado kinase (Tub-4/M10-2), dCyd kinase (AraC-8D), and both Ado kinase and dCyd kinase (AraC-8D-M10-5) have been reported previously [13, 24]. Secondary deficiencies in adenine phosphoribosyltransferase (APRTase) activity were inserted into the wild type, AraC-8D, and AraC-8D-M10-5 cell lines by selecting rare variants resistant to 100 μM 2,6-deaminopurine (DAP), a cytotoxic APRTase substrate, by techniques similar to those described by Ullman *et al.* [13] for the isolation of the Ara-C-8C cell line. All DAP-resistant CEM cell lines were demonstrated to be completely deficient in APRTase activity by the direct radiometric assay described initially by Iovannisci and Ullman [25]. The cell lines employed in this study were all deficient in the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase (HGPRTase), because the nucleoside kinase-deficient cells were generated for elucidating the pathways by which dAdo and dGuo were metabolized in human T cells [24]. The HGPRTase deficiency prevented the conversion of the two purine 2′-deoxyribonucleosides to ribonucleotides. Moreover, the HGPRTase deficiency is extremely useful in the metabolic studies described in this manuscript, since [^{3}H]ddAdo radiolabeled in the purine moiety cannot be converted to purine ribonucleotides. For the purposes of these investigations, the absence or presence of HGPRT is not pertinent to the metabolism or cytotoxicity of ddAdo, and therefore the HGPRTase-deficient parent of the nucleoside transport- and kinase-deficient cell lines can be considered the equivalent of a wild type cell line.

The CEM cells were propagated continuously in Dulbecco's modified Eagle's medium supplemented with 10% horse serum at 37° in a humidified 10% CO_2 atmosphere [13, 24]. Under these growth conditions, the doubling time of CEM cells was approximately 24 hr.

Incorporation of [^{3}H]*ddAdo by CEM cells.* To assay the rate of incorporation of ddAdo into wild type and mutant CEM cells, a modification of the method of Aronow *et al.* [26] was employed. Briefly, cells were harvested by centrifugation and resuspended at a density of 5×10^7 cells/mL in Dulbecco's modified Eagle's medium lacking serum but containing 20 mM HEPES and 20 μM EHNA [20]. One hundred microliters of transport medium containing 10 μM [^{3}H]ddAdo (10 μCi/mL) and 20 μM EHNA was laid over 150 μL of an inert oil mixture consisting of silicone oil (Catalog No. 17,563-3; Aldrich Chemical Co.) and paraffin oil (Catalog No. 0-119; Fischer Scientific) in a 94 : 6 ratio (density 1.03 g/mL) in a polypropylene Eppendorf centrifuge tube. Transport measurements were initiated by the swift addition of 100 μL of cells and terminated by sedimentation of the cells through the oil at 10,000 *g* for 25 sec. After aspiration of the upper radioactive layer, the surface of the oil was washed twice with 1.0 mL of water and aspirated. The cell pellet was lysed in 100 μL of 1% Triton X-100 and the radioactivity quantitated by liquid scintillation.

Growth rate determinations. The sensitivities of wild type and mutant CEM cells to growth inhibition by ddAdo were determined as described previously for CEM cells by Ullman *et al.* [13]. Briefly, cells at a concentration of 10^5 cells/mL were exposed to increasing concentrations of ddAdo in the presence of 10 μM EHNA. Two wells lacking ddAdo were maintained in parallel as controls. After 4–5 days of incubation with ddAdo, cells were enumerated in a Coulter Counter model ZM. The number of cells in each experimental well was subtracted from the number of cells in control wells and plotted as a percentage of the growth in the latter as a function of ddAdo concentration.

RESULTS

Phosphorylation of nucleoside analogs by dCyd kinase. Using the homogeneous human dCyd kinase enzyme, the ability of dCyd kinase to catalyze the phosphorylation of a variety of naturally occurring nucleosides and ddNs was assessed. These assays employed either [^{3}H]dCyd or [^{3}H]dAdo as phosphate

Table 1. Capacity of human deoxycytidine kinase to phosphorylate 2',3'-dideoxynucleosides

Substrate (mM)	dCyd kinase activity* (pmol/min)	
	Disc assay	HPLC assay
dCyd (0.1)	4.4 ± 0.8	2.6
ddCyd (0.2)	3.2 ± 0.2	3.5
dAdo (0.2)	26.0 ± 0.5	24.5
ddAdo (1.0)	<0.1 ± 0.2	<0.09
ddGuo (1.0)	<0.1 ± 0.3	<0.08

* dCyd kinase activities using 30 ng of enzyme were determined either with the Whatman DE81 filter disc assay or by high performance liquid chromatography on a Whatman Partisil-10-SAX column as described under Materials and Methods. Values reported for the disc assay are the means ± SEM of at least four determinations.

acceptors or [γ-^{32}P]ATP as the phosphate donor. As shown in Tables 1 and 2, purified dCyd kinase catalyzed the phosphorylation of dCyd, ddCyd, and dAdo, whereas the three purine ddNs, ddAdo, ddIno, and ddGuo, were not phosphorylated. The lack of phosphorylation of ddAdo could be attributed either to a failure of the dCyd kinase to recognize ddAdo as a substrate or to a selective loss of ddAdo phosphorylating capacity of the enzyme during the final steps of purification [14]. Therefore, a partially purified dCyd kinase fraction, one obtained after the DEAE-cellulose chromatography purification step [14], was analyzed for its ability to phosphorylate ddAdo. This cruder dCyd kinase preparation also was incapable of catalyzing the phosphorylation of the purine ddN (data not shown).

The kinetic parameters for the phosphorylation of dCyd, dAdo, and ddCyd were calculated by linear regression analyses of double-reciprocal plots (Table 3). dCyd was by far the most efficient substrate for dCyd kinase with a K_m value of 1.5 μM, whereas the K_m values of the enzyme for dAdo and ddCyd were 110 and 60 μM, respectively. However, the V_{max} value of dCyd kinase for dAdo was greater than those obtained for the cytosine-containing nucleosides. That dCyd, dAdo, and ddCyd are all phosphorylated by the same enzyme is supported by the observation that each was capable of inhibiting the phosphorylation of the others, as shown in Table 3. Double-reciprocal plots of the data demonstrated that the inhibition was competitive in nature (data not shown). That the K_i values did not correspond to the K_m values for phosphorylation (Table 3) has been reported previously [14]. Although the reason for these discrepancies is unclear, Sarup *et al.* [9] described the same phenomenon with dCyd kinase purified from human leukemic cells.

Inhibition of dCyd and dAdo phosphorylation by antiviral nucleoside analogs. Human dCyd kinase has a broad substrate specificity and can phosphorylate naturally occurring purine and pyrimidine nucleosides, although with differing V_{max} and K_m values [14]. To test whether anti-HIV analogs, many of which are currently commercially unavailable in radioactive form, were also phosphorylated by the purified dCyd kinase, these analogs were examined for their abilities to inhibit [^{3}H]dCyd and [^{3}H]-dAdo phosphorylation. As shown in Table 2, cytidine analogs, including dCyd, arabinosylcytosine (araCyt), and ddCyd, were inhibitors of human dCyd kinase with either 1 μM [^{3}H]dCyd or 30 μM

Table 2. Nucleoside analogs as inhibitors of deoxynucleoside phosphorylation and their relative phosphorylation rates by human dCyd kinase

Compounds	IC_{50}* (μM)		Relative phosphorylation rates†
	dAdo	dCyd	
dCyd	0.5	1	1
araCyt	5	410	1
ddCyd	20	670	1/3
Cytallene	8	95	1/10
D4C	85	ND‡	1/20
dAdo	30	>1000	3
ddAdo	>500	>1000	<1/200
ddGuo	>500	>1000	<1/100
ddINo	>1000	>1000	<1/100
Adenallene	>600	>600	<1/100
D4T	>800	ND	ND

The abilities of various nucleoside analogs to inhibit the phosphorylation of 30 μM dAdo and 1 μM dCyd by human dCyd kinase in the presence of 1 mM ATP:$MgCl_2$ and relative efficiencies of 100 μM nucleoside analog to accept [^{32}P] from 100 μM [γ-^{32}P]ATP were determined as described in Materials and Methods.

* The IC_{50} values are the concentrations of added nucleoside that give 50% inhibition of kinase activity compared to the control. The values are the means of at least two independent determinations.

† The relative values are the means of at least two separate determinations and the actual transfer to dCyd was approximately 8% in different experiments.

‡ Not determined.

Table 3. Kinetic parameters for substrates and inhibitors of human deoxycytidine kinase

Substrate	Inhibitors	K_m (μM)	V_{max} (nmol/min/mg)	K_i (μM)
dCyd		1.5	185	
dAdo		110	800	
ddCyd		60	220	
dCyd	ddCyd			4400
dAdo	ddCyd			83
dCyd	dAdo			3200

K_m, V_{max}, and K_i values were determined by linear regression analysis. Substrate concentrations employed were 0.5 to 10 μM for dCyd and 10 to 200 μM for ddCyd and dAdo.

[^{3}H]dAdo as phosphate acceptor, whereas purine ddNs, including ddAdo, ddIno, and ddGuo at concentrations in the millimolar range did not affect the rate or extent of dCyd or dAdo phosphorylation. The cytidine analogs were more effective inhibitors of dAdo phosphorylation, since dAdo is a much less efficient substrate than dCyd for dCyd kinase. Several modified nucleosides, including the acyclic compounds, cytallene and adenallene, and the olefinic ddN analogs, D4C and D4T, all of which are effective inhibitors of HIV replication, were also examined for their abilities to inhibit the phosphorylation of dCyd and dAdo. Whereas cytallene and D4C interfered with deoxyribonucleoside phosphorylation, neither adenallene nor D4T appeared to influence enzyme activity (Table 2).

The relative efficiencies of antiviral nucleosides to accept phosphate from [γ-^{32}P]ATP are also shown in Table 2. As expected, there was as good correlation between the capacities of the nucleosides to inhibit phosphorylation of dCyd and dAdo with their abilities to serve as phosphate acceptors. Moreover, although cytosine-containing nucleosides such as dCyd or araCyt were phosphorylated efficiently, dAdo was phosphorylated 3-fold more rapidly. No detectable phosphorylation of purine ddNs was observed with the purified dCyd kinase using this type of assay.

Uptake of ddAdo by CEM cells. To define the pathways by which ddAdo is transported and accumulated by intact T cells that are known to be suitable hosts for HIV infection [7, 17], the abilities of wild type and mutant CEM cells genetically deficient in nucleoside transport, Ado kinase, and/or dCyd kinase to accrue [^{3}H]ddAdo-generated radioactivity in the presence of 20 μM EHNA were compared. The results depicted in Fig. 1 revealed no significant differences among the rates by which wild type, nucleoside transport-deficient, and nucleoside kinase-deficient CEM cells accumulated [^{3}H]ddAdo *in situ*. A double mutant lacking both dCyd kinase and Ado kinase was also just as capable of incorporating [^{3}H]ddAdo as wild type cells. Neither 4-nitrobenzylthioinosine (NBMPR) nor dipyridamole (DPA), two potent inhibitors of the mammalian nucleoside transport system [27, 28], inhibited the rate of [^{3}H]ddAdo entry into wild type CEM cells (data not shown). Finally, the ability of

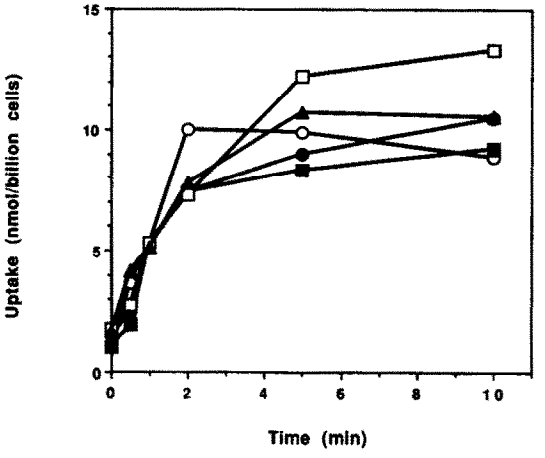

Fig. 1. [^{3}H]ddAdo uptake into CEM cells. The rate of [^{3}H]ddAdo accumulation by wild type (●), Ado kinase$^-$ (○), dCyd kinase$^-$ (■), Ado kinase$^-$/dCyd kinase$^-$ (□), and nucleoside transport$^-$ (▲) CEM cells was measured with the rapid sampling technique described in Materials and Methods. The assays were performed with 5 μM ddAdo and 20 μM EHNA.

wild type cells to take up [^{3}H]ddAdo at a concentration of 5 μM could not be inhibited effectively by nonradioactive Ado, dAdo, inosine, deoxyinosine, dCyd, thymidine, or ddIno, each present at concentrations of 500 μM. Thus, our data suggest that the translocation of ddAdo across the plasma membrane and the rate of intracellular accumulation of ddAdo-associated radioactivity are independent of the nucleoside transport system and the genetically and biochemically defined Ado and dCyd kinase activities.

To evaluate whether the Ado and dCyd kinase activities play any role in ddAdo phosphorylation *in vitro*, the abilities of cytosolic extracts of wild type and nucleoside kinase-deficient CEM cells to phosphorylate dCyd, dAdo, and ddAdo were compared. As expected, crude soluble extracts prepared from dCyd kinase-deficient CEM cells exhibited a very low capacity to phosphorylate dCyd and were 60% deficient in dAdo phosphorylating

Table 4. Deoxynucleoside kinase activities in extracts from wild type and mutant CEM cells*

Cells	Kinase activity (pmol/min/mg)			
	dCyd	dAdo	ddAdo	ddAdo +IMP
Wild type	94	290	25.5 ± 1.5	11
dCyd kinase$^-$	6	130	24.6 ± 1.6	4
Ado kinase$^-$	102	50	13.5 ± 2.0	3
Ado kinase$^-$/dCyd kinase$^-$	4	13	13.4 ± 2.0	4

* dCyd kinase activity was determined with 10 μM dCyd in the standard assay [11]. dAdo and ddAdo kinase activities were determined with 200 μM dAdo or ddAdo plus 10 μM EHNA with 30 or 120 μg extract, respectively. ddAdo phosphorylating activity was linear for only 20 min. For one series of experiments, 3 mM IMP was added to the ddAdo phosphorylation assay mixture. Values for dCyd and dAdo phosphorylation are the means of at least three measurements with the individual values differing from the mean by less than 15%. The values (means ± SD) for ddAdo phosphorylation were based on three determinations.

capacity (Table 4). Extracts prepared from Ado kinase-deficient and wild type cells phosphorylated dCyd at comparable rates, while dAdo phosphorylation was diminished by 80% in the mutant strain. The cell line deficient in both Ado kinase and dCyd kinase activities phosphorylated both dCyd and dAdo very poorly. Cytosolic fractions of wild type and dCyd kinase-deficient cells were equally capable of catalyzing the phosphorylation of ddAdo to the nucleotide level at virtually equivalent rates, but soluble extracts of the two mutant cell lines lacking Ado kinase manifested a significantly reduced ddAdo phosphorylating capacity (Table 4). Johnson and Fridland [29] have reported that the phosphorylation of ddIno can be catalyzed by a soluble 5′-nucleotidase activity using IMP as the phosphate donor. In an attempt to investigate if ddAdo phosphorylation is mediated by 5′-nucleotidase, the effect of 3 mM IMP on ddAdo phosphorylation by CEM cell extracts was evaluated. As shown in Table 4, 3 mM IMP inhibited ddAdo phosphorylation by 60–80%.

Growth sensitivities of CEM cells to ddAdo. Another method of assessing the role of dCyd kinase in the phosphorylation of ddAdo *in situ* is by comparing the growth sensitivities of wild type and dCyd kinase-deficient cells to ddAdo. These studies were also carried out in the presence of 10 μM EHNA to inhibit the deamination of ddAdo in ddIno. The data shown in Fig. 3 indicated that ddAdo could inhibit the growth of wild type and dCyd kinase-deficient cells to an equivalent extent. Moreover, cells defective in nucleoside transport were also as sensitive as wild type cells to the growth inhibitory effects of ddAdo, and neither 10 μM NBMPR nor 10 μM DPA ameliorated ddAdo toxicity toward wild type CEM cells (data not shown). Surprisingly, however, CEM cells lacking Ado kinase activity were approximately 5-fold less sensitive to the growth inhibitory effects of the ddN. Whereas the effective concentrations which inhibited the growth of wild type, nucleoside transport-deficient,

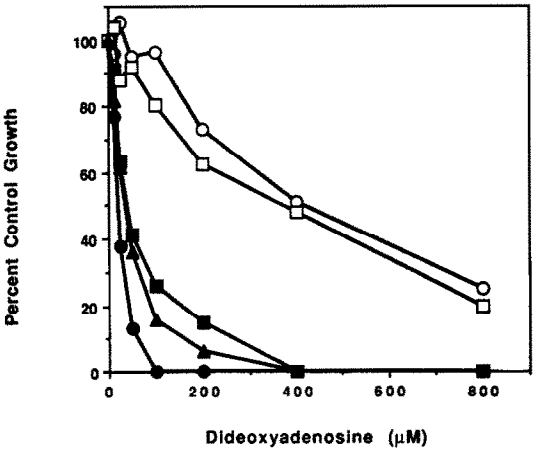

Fig. 2. Effects of ddAdo on the growth of wild type and mutant CEM cells. The growth inhibitory and cytotoxic effects of ddAdo on wild type (●), Ado kinase$^-$ (○), dCyd kinase$^-$ (■), Ado kinase$^-$/dCyd kinase$^-$ (□), and nucleoside transport$^-$ (▲) CEM cells were determined after 72 hr as described in Materials and Methods. EHNA to a concentration of 10 μM was added to all the cells. This experiment was repeated three other times with similar results.

and dCyd kinase-deficient cells were approximately 25–40 μM (EC_{50} value), the EC_{50} value for Ado kinase-deficient CEM cells for ddAdo was approximately 400 μM. The cell line that lacked both Ado kinase and dCyd kinase was as resistant as the Ado kinase deficient cell line to ddAdo.

Effect of APRTase mutation on ddAdo incorporation and toxicity. To eliminate the possibility that depurination of ddAdo contributed to incorporation or toxicity assessments, APRTase-deficient mutants of wild type, dCyd kinase, and dCyd kinase/Ado kinase deficient cells were generated and

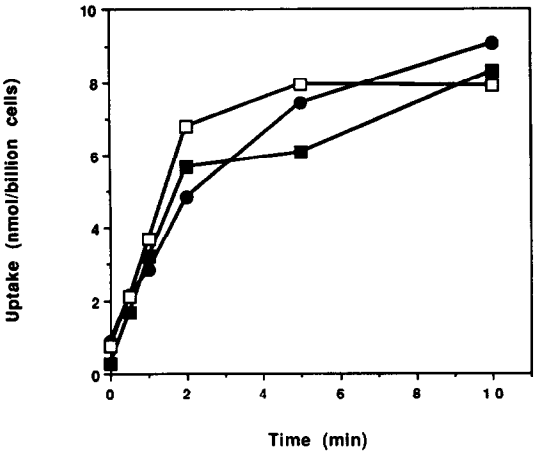

Fig. 3. [^{3}H]ddAdo incorporation into APRTase-deficient CEM cells. The rate of [^{3}H]ddAdo uptake into APRTase-deficient derivatives of wild type (●), dCyd kinase$^-$ (■), and Ado kinase$^-$/dCyd kinase$^-$ (□) cells was measured precisely as described for the experiment depicted in Fig. 1.

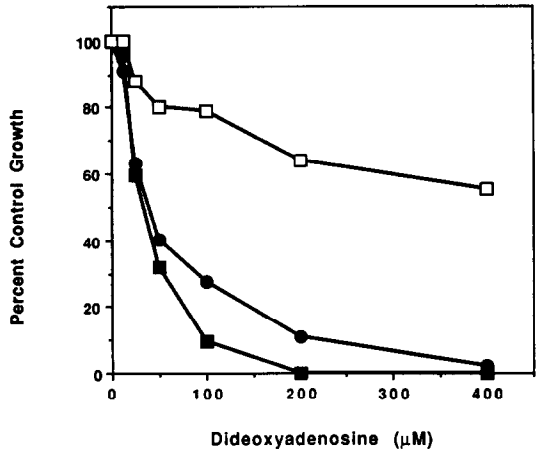

Fig. 4. Effects of ddAdo on the growth of APRTase-deficient CEM cells. The growth inhibitory and cytotoxic effects of increasing ddAdo concentrations on the APRTase-deficient derivatives of wild type (●), dCyd kinase$^-$ (■), and Ado kinase$^-$/dCyd kinase$^-$ (□) cells were determined as described for the experiment in Fig. 2.

compared with respect to their abilities to incorporate radiolabel from [^{3}H]ddAdo and to have their growth inhibited by ddAdo. As shown in Fig. 3, the three APRTase-deficient cell lines all incorporated [^{3}H]-ddAdo at comparable rates. These data provide an important genetic demonstration that measurements of [^{3}H]ddAdo incorporation in these experiments were not artifactually influenced by cleavage of the relatively labile *N*-glycosidic linkage of ddAdo. Moreover, the APRTase deficiency did not influence the sensitivity of the wild type or kinase-deficient cells to ddAdo toxicity, as shown in Fig. 4.

DISCUSSION

These investigations demonstrate that a homogeneously purified dCyd kinase enzyme from human leukemic spleen retained the capacity to recognize ddCyd as a substrate. Previously, several groups working with partially purified enzyme preparations have suggested that dCyd kinase is responsible for the phosphorylation of ddCyd [2, 4, 10, 11]. Starnes and Cheng [4] indicated that ddCyd is a substrate for both the cytosolic and mitochondrial dCyd kinases from human Molt/4 T cells with apparent K_m values of 180 and 120 μM, respectively. The V_{max} values of both Molt/4 enzymes for ddCyd are much less than those obtained for their preferred substrate, dCyd. Balzarini *et al.* [10, 11] also demonstrated that a partially purified dCyd kinase from Molt/4 cells could phosphorylate ddCyd with an apparent K_m value of 210 μM and a V_{max} 30% of that for dCyd. However, since the studies reported above were not performed with pure enzyme, the possibility that contaminant enzyme activities perturbed the kinetic results cannot be eliminated. Consistent with this premise is the observation that the 6000-fold purified human dCyd kinase employed in these studies had lower K_m values and higher V_{max} values for substrates than those obtained with the partially purified enzymes.

It should be noted that there was a considerable discrepancy between the K_i values observed for ddCyd, araCyt, and dAdo inhibition of dCyd phosphorylation and the K_m values observed for these substrates with the human dCyd kinase [14]. Similar results were obtained by Sarup *et al.* [9] and Balzarini *et al.* [10] with the partially purified enzyme. The reason for this unusual behavior is not known. Regardless of these kinetic properties, however, the results of these studies, coupled with the observations that dCyd kinase-deficient CEM cells are both insensitive to growth inhibition by ddCyd and incapable of phosphorylating [^{3}H]ddCyd to the nucleotide level *in situ* [13], demonstrate quite conclusively that dCyd kinase initiates the metabolism of ddCyd in cells of thymic origin.

The studies described herein were initiated in large part to determine if the substrate specificity of dCyd kinase included other ddNs that are known to be inhibitors of HIV proliferation. Previous studies with CEM cells convincingly demonstrated that dCyd kinase catalyzes the phosphorylation of the two deoxyribonucleosides, dAdo and dGuo, since CEM cells genetically deficient in dCyd kinase are less capable of phosphorylating exogenous dAdo and dGuo and are resistant to the cytotoxic effects of low concentrations of these compounds in the culture medium [24, 30]. The results of these investigations also demonstrate that dCyd kinase catalyzed the phosphorylation of the cytosine-containing anti-HIV nucleosides, cytallene and D4C, as well as ddCyd, araCyt, dAdo, and dGuo. However, the purified enzyme, as assessed by a variety of independent methods, did not catalyze the phosphorylation of the purine ddNs, ddAdo, ddIno, and ddGuo. The findings with ddAdo are discordant with previous conclusions arrived at by other workers. Two reports have indicated previously

that Ado kinase [5] and dCyd kinase [5, 6] activities from human thymic extracts had the capacity to phosphorylate ddAdo although the apparent K_m values for ddAdo phosphorylation were higher and the corresponding V_{max} values considerably lower than those of the natural substrates. Moreover, incompletely purified Ado kinase and dCyd kinase preparations from CEM cells were also reported to be capable of catalyzing ddAdo phosphorylation [8]. Recently, Sarup *et al.* [9] have performed an extensive kinetic study with a dCyd kinase preparation that was approximately 20% pure. Although their results on purine deoxyribonucleoside phosphorylation generally agree with those reported here, Sarup *et al.* [9] did observe minute amounts of purine ddN phosphorylation by the partially pure enzyme, as well as a 10-fold lower V_{max} value for dCyd. The discrepancies in substrate specificity between our virtually homogeneous dCyd kinase and the partially purified enzyme characterized by Sarup *et al.* [9] remain to be resolved. To minimize methodological differences between this and the previous studies, we followed exactly the protocol of Johnson *et al.* [6] in preparing CEM cell extracts and were unable to demonstrate significant differences in ddAdo phosphorylation capability among wild type and Ado kinase- and dCyd kinase-deficient cells. However, there was a significant reduction in the ddAdo phosphorylating capacity of soluble extracts of Ado kinase-deficient cells. The low residual activity found in Ado kinase-deficient mutants may be due to other presently unidentified kinase activities.

Intact CEM cells genetically altered in nucleoside transport or deoxynucleoside kinase activities were employed to probe the biochemical determinants involved in ddAdo translocation and uptake. Nucleoside transport-deficient cells were just as capable as wild type CEM cells in accumulating exogenous ddAdo (Fig. 1) and were just as sensitive to growth inhibition by ddAdo in the presence of 10 μM EHNA (Fig. 2). Moreover, neither NBMPR nor DPA influenced ddAdo toxicity toward or ddAdo accumulation by wild type cells. Therefore, in contrast to ddCyd which is transported via both nucleoside transporter-dependent and nucleoside transport-independent routes by CEM cells [13], ddAdo permeation across the plasma membrane or CEM cells did not appear to be mediated by the relatively nonspecific mammalian nucleoside transport system [31]. Measurements of ddAdo uptake by intact wild type and mutant cells suggested that ddAdo was accumulated by cells genetically deficient in Ado kinase, dCyd kinase, or both enzymes at the same rate as by wild type cells. These studies were carried out in the presence of 10 μM EHNA to prevent ddAdo deamination to ddIno and in HGPRTase-deficient cells to prevent any conversion of the radiolabel in the adenine ring into purine ribonucleotides via the sequential actions of residual adenosine deaminase, purine nucleoside phosphorylase, and HGPRTase. Moreover, APRTase-deficient derivatives of wild type and mutant CEM strains incorporated [^{3}H]ddAdo at comparable rates to each other and to their respective parental cell lines, indicating that the radiolabel in the purine ring of [^{3}H]ddAdo was not incorporated subsequent to glycosidic cleavage and phosphoribosylation.

Previous studies measuring ddAdo metabolites in kinase-deficient CEM cells exposed to ddAdo has suggested that dCyd kinase might play a primary role in ddAdo phosphorylation. Although Carson *et al.* [7] demonstrated that CEM cells deficient in both Ado and dCyd kinase activities accumulated as much ddATP as wild type cells, dCyd kinase deficient cells degraded ddATP to a greater extent than wild type parental cells. Moreover, Johnson *et al.* [8] demonstrated that ddATP accumulation was much more efficient in wild type cells than in cells lacking either dCyd kinase or Ado kinase, although significant ddATP incorporation was observed in CEM cells deficient in both enzymes. The discrepancies between the present and previous studies can be attributed to the much shorter time course employed in our ddAdo accumulation experiments. Agarwal *et al.* [32] have reported that H9 T cells, Namalwa B cells, and peripheral blood mononuclear cells of human origin can all accumulate ddAdo-associated radioactivity within 10 min to levels an order of magnitude higher than that of the extracellular medium. This accumulation was not associated with ddAdo phosphorylation to ddATP [32]. Thus, it is likely that our measurement over short time intervals reflected the relative abilities of wild type and genetically altered CEM cells to take up ddAdo into nonphosphorylated metabolites. Interestingly, dCyd kinase deficiency did not ameliorate the growth sensitivity of CEM cells toward ddAdo in the presence of 10 μM EHNA, whereas Ado kinase-deficient cells were 5-fold less sensitive to the toxic effects of the ddN. Thus, our observations made *in situ* with intact cells and *in vitro* with purified enzyme suggest that Ado kinase mediates the toxicity of ddAdo in the presence of EHNA by phosphorylating the ddN.

A mitochondrial nucleoside kinase that phosphorylates inosine and guanosine has been described, but no evidence exists that this enzyme is capable of phosphorylating exogenous ddNs [33, 34]. Several groups have suggested that a cytoplasmic 5'-nucleotidase activity is capable of operating as a phosphorylating enzyme with inosine and guanosine as substrates [35, 36], and Johnson and Fridland [29] have shown recently that this enzyme can directly catalyze the phosphorylation of ddIno to ddIMP using IMP as the phosphate donor. Addition of 3 mM IMP to the ddAdo phosphorylation assay gave approximately 60–80% inhibition of the activity, suggesting that the ddAdo phosphorylation assay was not primarily measuring this phosphotransferase activity. The reason for the inhibitory effect of IMP on ddAdo phosphorylation is not known but may be a form of product inhibition of Ado kinase.

In summary, the pathways by which ddAdo and ddCyd are converted to the triphosphate level in CEM cells appear to be independent. ddCyd is translocated via the nucleoside transporter and is phosphorylated exclusively via dCyd kinase, whereas ddAdo permeates the plasma membrane by a route independent of the nucleoside transporter and does not appear to be a substrate for the human dCyd

kinase *in vitro* or *in situ*. Rather, our results indicate that Ado kinase may be involved in the phosphorylation of ddAdo. Although, the clinical utility of ddAdo for the treatment of AIDS has been limited by its toxicity and its rapid deamination to ddIno [37, 38], a substantial number of new purine and pyrimidine analogs are currently under investigation. The independence of the pathways for the conversion of extracellular pyrimidine and purine ddNs to intracellular ddNTPs as delinated by these studies on pure dCyd kinase enzyme and somatic cell mutants suggests that these two classes of compounds could potentially be exploited in combination to improve their anti-HIV efficacy and reduce the risk of emergence of drug-refractory virus.

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