

Molecular and Biochemical Mechanisms of Fludarabine and Cladribine Resistance in a Human Promyelocytic Cell Line¹

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ABSTRACT

2F-Adenine arabinoside (fludarabine, Fara-A) and 2-chloro-2'-deoxyadenosine (cladribine, CdA) are nucleoside analogues with antineoplastic activity *in vitro* and *in vivo*. Lack of clinical resistance between CdA and Fara-A has been demonstrated in patients with chronic lymphocytic leukemia (G. Juliusson *et al.*, *N. Engl. J. Med.*, 327: 1056–1061, 1992). To clarify the differences in mechanism of resistance to CdA and Fara-A *in vitro*, we developed two stable, resistant cell lines, HL60/CdA and HL60/Fara-A, by exposure to increasing concentrations of analogues over a period of 8 months. Resistant cells tolerated >8000 and 5-fold higher concentrations of CdA and Fara-A, respectively. The specific activity of the nucleoside phosphorylating enzyme (using deoxycytidine as substrate) in cell extracts from HL60/CdA and HL60/Fara-A mutants was about 10 and 60%, respectively, compared with the parental cell line. Western blot analysis using a polyclonal antibody showed no detectable deoxycytidine kinase (dCK) protein in CdA-resistant cells, whereas in Fara-A-resistant cells, it was at the same level as in the parental cells. The mitochondrial enzyme deoxyguanosine kinase was not altered in resistant cell lines. The HL60/CdA cells showed cross-resistance to 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, Fara-A, arabinofuranosyl cytosine, difluorodeoxyguanosine, and difluorodeoxycytidine toxicity, most likely because of the decreased phosphorylation of these analogues by dCK.

Using real-time quantitative PCR, the mRNA levels of dCK and cytosolic 5'-nucleotidase (5'-NT), a major nucleoside dephosphorylating enzyme, were measured. It was shown that the dCK mRNA levels in both CdA- and Fara-A resistant cells were decreased in parallel with the activity. The expression of 5'-NT mRNA was not significantly elevated in CdA- and Fara-A resistant cells, as compared with the parental cells. Ribonucleotide reductase maintains a balanced supply of deoxynucleotide triphosphate pools in the cell and may also be a major cellular target for CdA and Fara-A nucleotides. Except for the deoxycytidine triphosphate level, the intracellular deoxynucleotide triphosphate pools were significantly higher in Fara-A-resistant cells compared with the parental cell line. This might be a consequence of mutation or altered regulation of ribonucleotide reductase activity and may explain the 2–5-fold cross-resistance to several nucleoside analogues observed with HL60/Fara-A cells. It is likely that the resistance for CdA was mainly attributable to a dCK deficiency, and Fara-A-resistant cells might have another contributing factor to the resistance beyond the dCK deficiency.

INTRODUCTION

The antimetabolites Fara-A³ and CdA are purine nucleoside analogues with activity in lymphoproliferative disorders. CdA and Fara-A have many common characteristics with respect to their structures and

metabolism, but their differences in the mechanism of action give them unique clinical activity. CdA is very effective in hairy cell leukemia, with ~85% complete response; it has also shown efficacy in other lymphoproliferative disorders, such as chronic lymphocytic leukemia, low-grade non-Hodgkin's lymphomas and Waldenström's macroglobulinemia, as well as in childhood acute myelogenous leukemia (1). CdA also has impressive clinical activity against multiple sclerosis (2). Fara-A has proved to be effective in various lymphoproliferative disorders and as a single agent in therapy for chronic lymphocytic leukemia (3), low-grade non-Hodgkin's lymphomas (4), and other hematological malignancies (5). Fara-A has also been shown to have synergistic effects in combination with other chemotherapeutic drugs, *e.g.*, ara-C, cyclophosphamide, and also in combination with radiation (reviewed in Ref. 6). Implications for different mechanisms of resistance for CdA and Fara-A is that patients with chronic lymphocytic leukemia treated previously with Fara-A respond to CdA (7).

CdA and Fara-A require intracellular phosphorylation for their cytotoxic actions. dCK is the enzyme responsible for the initial phosphorylation, and the cytoplasmic enzyme 5'-NT is responsible for the dephosphorylation of these nucleoside analogues (8). However, these drugs as well as 9-β-D-arabinofuranosylguanine are substrates for the mitochondrial enzyme dGK (9). In a recent study, it was shown that the overexpression of dGK as a fusion protein in a human pancreatic cancer cell line increases the sensitivity to CdA and 9-β-D-arabinofuranosylguanine (10). Whether the activity of dGK contributes significantly to the cytotoxicity of these analogues in hematological malignancies is not clear.

In replicating cells, CdATP inhibits DNA polymerase as well as the enzyme ribonucleotide reductase, causing deoxynucleotide triphosphate pool imbalance (11). CdATP is also incorporated into DNA, producing strand breaks and inhibition of DNA synthesis (12). In resting cells, CdATP interferes with the proper repair of DNA strand breaks, which activates poly(ADP-ribose) synthetase, and NAD⁺ is depleted, leading to apoptosis (13, 14). The triphosphate of Fara-A, Fara-ATP, inhibits DNA synthesis (15, 16) and ribonucleotide reductase (17). Fara-A is also incorporated into RNA (18). Recently, it has been shown that CdATP as well as Fara-ATP can cooperate with cytochrome *c* and induce caspase-3 activation and the caspase proteolytic cascade, leading the cell to apoptosis (19, 20).

Resistance to chemotherapeutic agents is one of the major problems in the treatment of leukemia. The mechanisms behind CdA and Fara-A therapy failure have not been well documented, but deficiency of dCK activity has been encountered as a cause of resistance to these drugs in cultured cells. In addition to decreased dCK activity, other biochemical mechanisms for resistance could be altered intracellular pools of deoxynucleotides, increased drug inactivation by 5'-NT, and decreased nucleoside transport into the cells. Reductions in dCK activity led to CdA resistance in WIL2 human B lymphoblastoid cells, an L1210 murine leukemia cell line, and CCRF-CEM cells (21, 22). Studies in chronic lymphocytic leukemia patients also demonstrated that dCK levels were significantly higher, whereas 5'-NT levels were significantly lower in CdA responders than in nonresponders (8, 23). Also, increased mRNA expression and activity of

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³ The abbreviations used are: Fara-A, fludarabine, 2F-adenine arabinoside; CdA, cladribine, 2-chloro-2'-deoxyadenosine; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; 5'-NT, 5'-nucleotidase; ara-C, cytarabine, arabinofuranosyl cytosine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; HPLC, high-performance liquid chromatography.

5'-NT has been shown to induce CdA resistance in HL60 cells (24). Ribonucleotide reductase is a primary target of CdA and Fara-A nucleotides; they inhibit the enzyme and decrease cellular deoxynucleotide pool levels (5). A mechanism for CdA and Fara-A resistance other than decreased activity of dCK and increased activity of 5'-NT could be a mutation or altered regulation of ribonucleotide reductase.

We report here on characterization of two cell lines resistant to CdA and Fara-A *in vitro*, selected by a protocol mimicking the appearance of clinical resistance. To achieve this goal, we analyzed the intracellular metabolism of CdA and Fara-A, and the activity of the phosphorylating enzymes dCK, dGK, and thymidine kinase were determined. The mRNA expression of dCK and 5'-NT was also quantitated by real-time quantitative PCR analysis. Furthermore, the intracellular accumulation of the respective nucleotides as well as the intracellular deoxynucleotide pools were determined. A preliminary report of this study has been presented at the American Association for Cancer Research meeting (25).

MATERIALS AND METHODS

Chemicals. Deoxyadenosine, deoxycytidine, ara-C, hydroxyurea, tubercidin, and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). EHNA was purchased from Burroughs Wellcome Co., and SDS was purchased from KEBO lab (Stockholm, Sweden). Fara-A was a gift from Dr Ze've Shaked (Berlex, Alameda, CA). CdA and CdAMP were synthesized by Dr. Zygmunt Kazimierzczuk at the Foundation for the Development of Diagnostics and Therapy (Warsaw, Poland). Etoposide and teniposide were provided by Bristol-Myers Squibb (Bromma, Sweden). Difluorodeoxycytidine and vincristine were obtained from Lilly (Stockholm, Sweden). 2-Chloro-2'-arabino-fluoro-2'-deoxyadenosine was a gift from Dr. Howard Cottam (University of California, San Diego, CA). 9- β -D-Arabinofuranosylguanine was from R. I. Chemical (Orange, CA). Daunorubicin was purchased from Rhone-Poulenc Rorer (Bristol, England), 5-fluorouracil from Roche (Stockholm, Sweden), and mitoxantrone (Novantrone) from Lederle/Cyanamid (Stockholm, Sweden). Difluorodeoxyguanosine was from Lilly. [5-³H]Deoxycytidine (16.7 Ci/mmol), [8-³H]CdA (4 Ci/mmol), [8-³H]Fara-A (15 Ci/mmol), [methyl-³H]TTP tetraammonium salt (30 Ci/mmol), and [2,8-³H]2'-deoxyadenosine 5'-triphosphate tetraammonium salt (15 Ci/mmol) were purchased from Moravak Biochemicals (Brea, CA). [5-H]Ara-C (33 Ci/mmol) and [6-³H]thymidine (24 Ci/mmol) were obtained from Amersham (Little Chalfont, England). CdATP was synthesized by Sierra Bioresearch (Tucson, AZ) and provided by Dr. William Plunkett (University of Texas M. D. Anderson Cancer Center, Houston, TX). NH₄H₂PO₄ was purchased from Merck (Darmstadt, Germany). RPMI 1640, heat-inactivated FCS, L-glutamine, and penicillin-streptomycin were from Life Technologies (Paisley, United Kingdom). DNA polymerase I (Klenow fragment) was purchased from Amersham (Stockholm, Sweden). All reagents for PCR reactions were purchased from Perkin-Elmer (Foster City, CA).

Cell Specimens. The HL60 cell line was originally described by Gallagher *et al.* (26). Cells were subcultured twice weekly in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine at 37°C in a humidified air atmosphere containing 5% CO₂ and routinely tested for *Mycoplasma* contamination. CdA and Fara-A resistances were induced in the HL60 cells by exposure to increasing concentrations of the drugs over a period of 8 months. At intervals of 2 weeks, the concentrations of the drugs were increased with 5 nM increments until concentrations of 50 nM CdA and 300 nM Fara-A were reached and then with 10 nM for CdA and 50 nM for Fara-A increments until the final concentrations of 150 nM CdA and 2000 nM of Fara-A. The CdA and Fara-A resistances were maintained by adding relevant concentrations of respective drugs when cells were subcultured.

The number of cells in the samples and the median cell volume of the samples were determined by a Coulter Multisizer (Coulter Electronics, Luton, United Kingdom). The number of cells in G₁, S, and G₂+M phases of the cell cycle were determined by 4,6-diamidino-2-phenylindole staining using a PAS II cytometer (Partec, Münster, Germany), which was excited at 365 nm, and

the fluorescence was measured at >435 nm. The multicycle program (Phoenix Flow System, San Diego, CA) was used for histogram analysis.

Before conducting any experiments, the cells were cultured in drug-free medium for three passages. Then cells were cultured during logarithmic phase (about 0.8–1.3 $\times 10^6$) over a period of 3 days, centrifuged 5 min at 1200 \times g, and washed twice with prewarmed RPMI 1640 containing 25 mM HEPES (RPMI 1640-HEPES; Life Technologies, Inc.). The cells were suspended in the same medium to produce 2 $\times 10^6$ cells/ml.

Cytotoxicity Assay. Drug sensitivity was assessed using MTT at 37°C for 72 h as described previously (27). In brief, cells were suspended in medium to a concentration of 3 $\times 10^5$ cells/ml. Then 100- μ l aliquots of this suspension were dispensed into 96-well, round-bottomed microtiter plates, which already contained 5 μ l of drug dilutions in triplicate (9–18 concentrations). To measure the cytotoxicity to deoxyadenosine, cells were preincubated with 10 μ M EHNA for 1 h to inhibit degradation by adenosine deaminase. Wells containing no drugs were used for control of cell viability. After the cells were incubated, 10 μ l of MTT solution (5 mg/ml tetrazolium salt) were added to each well, and the plates were incubated further for 4 h in 37°C. The formazan salt crystals were dissolved with 100 μ l of 10% SDS in 10 mM HCl solution overnight in 37°C. The absorbance of the wells was measured at the wavelength of 540 nm with reference at 650 nm by an ELISA plate reader (Labsystems Multiscan RC, Helsinki, Finland). Cell survival in a well was expressed as a percentage compared with the growth in control wells.

Drug Accumulation Studies. To study the accumulation of CdA and Fara-A nucleotides in resistant cells, exponentially growing cells in RPMI 1640-HEPES were exposed to 1 μ M CdA or 20 μ M Fara-A for 2 h. The cells were centrifuged at 1200 \times g for 5 min and washed two times with cold PBS, and the intracellular accumulation of nucleotides was analyzed as described below.

HPLC Analysis of CdA and Fara-A Nucleotides. CdA nucleotides were extracted and analyzed according to a method described earlier by Reichelova *et al.* (28). Briefly, 200 μ l of ice-cold 0.4 M perchloric acid containing 0.08 M triethylammonium phosphate were added to the cell pellet (10–20 $\times 10^6$ cells), mixed, and brought to pH 6.2 by addition of 100 μ l of ice-cold 1.2 M KOH/0.5 M NH₄H₂PO₄. After centrifugation at 13,100 \times g for 5 min at 4°C, a 100- μ l aliquot of the supernatant was injected into the HPLC column directly. The column used was an Ultrasphere ODS (250 \times 4.6 mm, 5 μ m; Beckman Instruments, Fullerton, CA) equipped with the Guard Pak precolumn (Bondapak C₁₈; Millipore, Milford, MA). The mobile phase consisted of triethylammonium phosphate buffer (0.08 M, pH 6.1) and methanol (89:11, v/v). The elution was carried out at flow rates of 1.5 ml/min at the ambient temperature (22°C). The temperature of the autosampler was maintained at 8°C. The concentration of the CdA nucleotides was determined at 265 nm by comparing the peak area of the CdA nucleotides in the cells to those of the standard substances. The amount of Fara-ATP was determined by extracting the cells as described above and analyzed as described below.

Determination of Deoxynucleotide Triphosphate Pools. For deoxynucleotide triphosphate measurements, cells were subcultured in RPMI 1640 with glutamine but without penicillin and streptomycin for at least two passages to prevent the inhibition of DNA-polymerase reaction. Cells were collected during logarithmic phase (0.8–1.3 $\times 10^6$), washed twice with ice-cold PBS, and counted, and 1–2 $\times 10^6$ cells were extracted overnight with 100 μ l of 70% methanol at –20°C. After subsequent evaporation, cell extracts were reconstituted in MilliQ water, and deoxynucleotide triphosphate pools were determined using DNA-polymerase (Klenow fragment) and synthetic oligonucleotide template primers according to Sherman and Fyfe (29), with minor modifications. The concentrations of template-primers for measurements of dCTP and dGTP pools were kept at 0.1 μ M to decrease the background. The effectiveness of measurements was further improved twice using elution of ³H-labeled oligonucleotides from DEAE-paper with 2 M NaOH (60 min shaking at room temperature) and counting in Ultima Gold (Packard) as described by van Moorsel *et al.* (30).

Intracellular deoxynucleotide triphosphate pools were also measured according to a HPLC method described previously (31). In brief, nucleotides were extracted with 0.4 M perchloric acid and neutralized with 1.2 M KOH/0.5 M NH₄H₂PO₄ and centrifuged, and the supernatant was collected. Then the ribonucleotides were removed from the extracts using periodate oxidation described previously by Griffing *et al.* (11). The deoxynucleotide triphosphates were measured by HPLC using a Partisil-10 SAX anion exchange column

(250 × 4 mm; Whatman), with gradient elution. The amount of each deoxynucleotide triphosphate pool was calculated by comparing the area under curve/height to a standard.

Measurement of dCK, dGK, and Thymidine Kinase Activities. Cells were suspended at 10⁶ cells/100 μl in an extraction buffer containing 50 mM Tris-HCl (pH 7.6), 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.5% NP40. The suspended cells were then frozen and thawed repeatedly three times and centrifuged at 11,800 × g in an Eppendorf centrifuge for 5 min at 4°C to remove cell debris. The supernatant was collected and used as a source of protein for the enzyme assays. Substrates were deoxycytidine (10 μM), CdA (50 μM), and thymidine (10 μM), with specific radioactivities of 500-1000 cpm/pmol. For each substrate, the concentration was consistently chosen to be 10 times higher than the K_m for the principle kinase phosphorylating the respective substrate. The assays were initiated by the addition of 2-3 μg of protein to a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 5 mM ATP, 4 mM DTT, 10 mM sodium fluoride, and substrates in a total volume of 25 μl. After incubation at 37°C for 15 and 30 min, 10-μl aliquots were withdrawn and spotted on Whatman DE81 papers. Filters were then washed as described by Spasokoukotskaja *et al.* (32), eluted by 50 μl of 0.4 M perchloric acid, and counted in 3 ml of Eosincint scintillation fluid in a liquid scintillation counter (RackBeta; LKB Wallac, Turku, Finland). The conditions that maintained linear reaction rates were determined in preliminary experiments. The specific activity of the enzymes was expressed in pmol of nucleoside phosphorylated by extract from 1 million cells during 1 min. The activity of dGK was determined by using CdA in the presence of 1 mM deoxycytidine to block the dCK enzyme.

Uptake, Phosphorylation, and DNA Incorporation of the Analogues. Cells (10⁶ cells in 500 μl of RPMI-HEPES) were incubated at 37°C with one of the following isotopes: thymidine (1 μCi, 0.08 μM) and deoxycytidine (1 μCi, 0.12 μM) for 30 min, with ara-C (1 μCi, 0.06 μM), CdA (0.3 μCi, 0.15 μM), and Fara-A (1 μCi, 0.13 μM) for 60 min. Labeling was terminated by cooling the samples to 0°C in an ice bath. The cells were washed twice with ice-cold PBS and extracted with 200 μl of ice-cold 0.4 M perchloric acid. After 30 min on ice, samples were centrifuged for 5 min at 11,800 × g in an Eppendorf centrifuge, and the radioactivities of acid-soluble nucleotide fractions were counted as described above. Acid-insoluble precipitates were washed twice with 0.4 M perchloric acid, and then nucleic acids were hydrolyzed by 200 μl of 0.4 M perchloric acid at 90°C for 30 min. Samples were cooled and centrifuged at 11800 × g, and the amount of radioactivity incorporated into nucleic acid was determined. The results were presented as pmol of labeled compound incorporated per million cells/hour.

Western Blotting. The level of dCK was determined in the HL60 cell extract by the Western blot method using a peptide anti-dCK antibody as described previously (33). For the determination of dGK protein levels, a similar assay was developed. Recombinant dGK (34) was used to immunize rabbits; dGK-specific antibodies were collected and affinity purified as described.⁴ Approximately 40 μg of crude cell extract were analyzed, and the protein bands were detected by the enhanced chemiluminescence immunodetection system as described by the suppliers (Amersham International, Buckinghamshire, United Kingdom).

Protein Determination. The protein content in cell extracts was determined according to the method of Lowry *et al.* (Ref. 35; DC protein assay; Bio-Rad Laboratories, Hercules, CA).

RNA Extraction and Reverse Transcription-PCR Analysis. For RNA extraction, 1 × 10⁷ cells were grown for each cell line and centrifuged to collect the pellet. RNA extraction was performed according to the manufacturer's instructions (RNeasy Midi Handbook; Qiagen, KEBO Lab, Spånga, Sweden). Then cDNA was synthesized from ~5 μg of total RNA in a 100-μl reaction mixture according to the manufacturer's instructions using an RNA PCR kit (GeneAmp; Perkin-Elmer) with random hexamers. To amplify the cDNA, 0.05 μg of the reversed transcribed cDNA from cell lines were subjected to 35 cycles of PCR in 50 μl of 1× PCR buffer, 2 mM MgCl₂, 0.8 mM each of dATP, dCTP, dGTP, and dTTP, 0.1 mM primers, and 0.625 unit of Taq DNA polymerase. Each cycle consisted of denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min in a DNA thermal cycler (Perkin-Elmer). Primers

for dCK were: forward, 5'-ACACCATGGCCACCCCGCCCAAGAGAAGCT-3' and reverse, 5'-CACGGATCTCACAAAGTACTCAAAAACTCTTT-3'. 5'-NT primers were: forward, 5'-TAAGCATGCCCTGAAAAAGTATCG-3' and reverse, 5'-GGAATCACCAAAAAAGTTCGCC-3'. Samples of each dCK and 5'-NT PCR product (18 μl) were electrophoresed in 1.5% agarose gel and photographed as ethidium bromide fluorescent bands. The PCR products were sized by a fX174 RF DNA/*Hae*III fragment (Life Technologies, Inc.). The PCR procedure was performed at least three times for each sample.

Real-Time Quantitative PCR. Real-time quantitative-PCR was earlier described by Gibson *et al.* (36). Primers and the Taqman probes for β₂-microglobulin, dCK, and 5'-NT were designed using the primer design software Primer Express (Perkin-Elmer Applied Biosystems). Tetrachloro-6-carboxy-fluorescein was chosen as reporter dye for 5'-NT and β₂-microglobulin and 6-carboxy-fluorescein for dCK. β₂-Microglobulin was used as internal control. Primers were made by DNA Technology A/S (Aarhus, Denmark), and the probes were made by Perkin-Elmer. Forward primer for β₂-microglobulin was 5'-ACTGGTCTTTCTATCTCTTGACTACTACTGA-3', and reverse primer was 5'-AGTCACATGGTTCACACGGC-3'. dCK primers were: forward, 5'-CCACCCCGCCCAAGAG-3'; and reverse, 5'-GATTTCTTGATGCGGGTCC-3'. The forward and reverse primers to quantify 5'-NT were 5'-AGCCTGTTTCGAGTGGC-3' and 5'-GGTCAGCATAACGCATCACTTG-

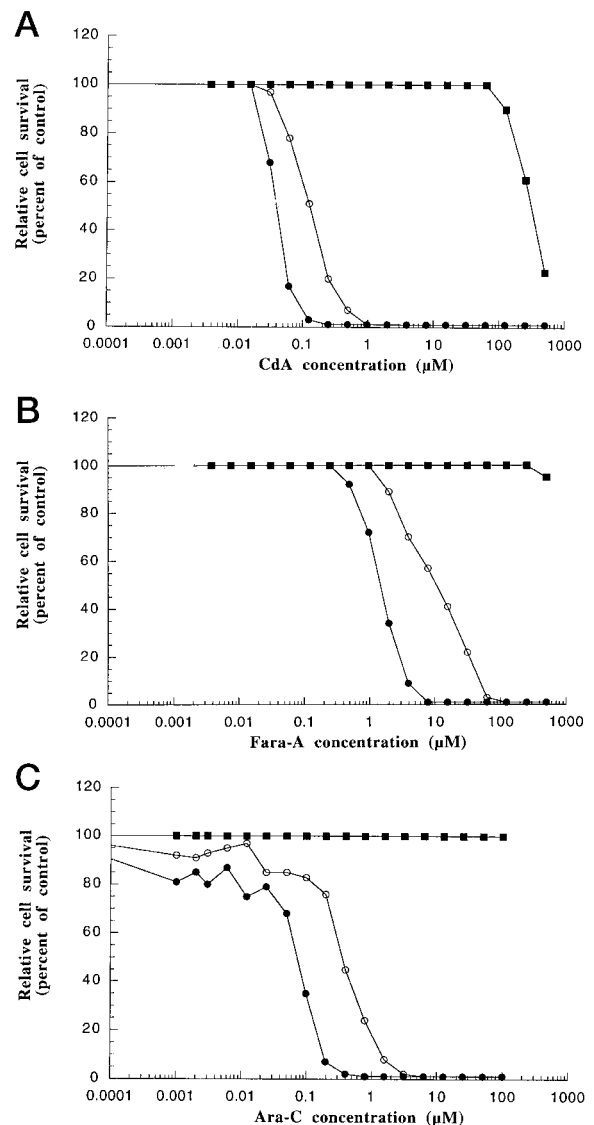


Fig. 1. Dose-response curves assessed by MTT, cytotoxicity assay of HL60 cells (●) and HL60 cells resistant to CdA (■) and Fara-A (○). Cells were incubated for 72 h with different concentrations of analogues, and cell survival was measured.

⁴ M. Jüllig, and S. Eriksson, manuscript in preparation.

Table 1 Cytotoxicity (IC_{50}) data and resistance factors (Rf) of wild-type, CdA, and Fara-A-resistant HL60 cells

The sensitivity of mutant and parental cell lines to different drugs was monitored by the MTT assay. Cells were incubated with drugs, and MTT was added to determine IC_{50} s, the concentration of drug tolerated by 50% of cells.

	HL60/wt	HL60/CdA		HL60/Fara-A	
	IC_{50} , μM	IC_{50} , μM	Rf	IC_{50} , μM	Rf
Antimetabolites					
CdA	0.041	338	8244	0.117	2.9
Fara-A	1.9	>500 ^a	>200	9.7	5.3
2-Chloro-2'-arabino-fluoro-2'-deoxyadenosine	0.037	>500	>10000	0.124	3.4
Ara-C	0.064	>100	>1500	0.315	4.9
Diffuorodeoxycytidine	0.067	79.8	1191	0.129	1.9
9- β -D-Arabinofuranosylguanine	166	289	1.7	275	1.7
Diffuorodeoxyguanosine	1.8	>50	>25	3.4	2.0
Deoxyadenosine (+ 10 μM EHNA)	2.8	21	7.5	5.5	2.0
5-Fluorouracil	29	29	1.0	21	0.7
Nucleoside transport inhibitor					
Tubercidin	0.273	0.240	0.9	0.296	1.1
Ribonucleotide reductase inhibitor					
Hydroxyurea	117	187	1.6	250	2.1
Topoisomerase interactive agents					
Teniposide	0.16	0.33	2.1	0.16	1.1
Etoposide	0.41	1.79	4.3	0.49	1.0
Anthracycline and related compounds					
Mitoxantrone	0.08	0.15	1.9	0.09	1.2
Daunorubicin	0.20	0.18	0.9	0.20	1.0
Vinca alkaloids					
Vincristine	0.05	0.05	1.1	0.06	1.0
Taxanes					
Paclitaxel	0.003	0.003	1.0	0.003	1.0

^a The highest concentration used in the assay.

3', respectively. Sequences for the Taqman probes were: $\beta 2$ -microglobulin, 5'-TCACCCCTACTGAAAAAGATGAGTATGCC-3'; dCK, 5'-TGC-CCGTCTTCTCAGCCAGCTCT-3'; and 5'-NT, 5'-CCCGGCAGAC-CCTTTTGCCAG-3'.

The PCR reaction mixture (25 μ l) contained TaqMan buffer, 2.5 mM $MgCl_2$, deoxynucleotide triphosphates (0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, and 0.4 mM dUTP), 0.32–0.5 μM primers, 0.1–0.2 μM probe, 0.625 unit of AmpliTaq Gold, 0.25 unit of uracil-N-glycosylase, and 50 ng of cDNA. The amplification consisted of 2 min at 50°C, 10 min at 95°C, and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C. The TaqMan probe is cleaved by the exonuclease activity of the Taq polymerase, separating the

fluorogenic reporter dye from the quencher and the fluorescence increases proportionally to the amount of PCR product. Reactions were performed, and data were collected by the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Results are expressed as the ratio of 5'-NT or dCK and $\beta 2$ -microglobulin.

RESULTS

Development of Resistant Cell Lines. Cell lines resistant to CdA and Fara-A were developed by adaptation of the HL60 cells to the drugs over a period of 8 months. HL60/CdA and Fara-A resistant cells were continuously cultured in medium containing 150 nM CdA and 2000 nM Fara-A, respectively. All cell lines have maintained stable resistance to the respective drug for more than 20 cell passages in the absence of drugs, as determined by the cytotoxicity assay and dCK measurement in cell extracts (data not shown).

Drug Sensitivity Measurements. The sensitivity of resistant cell lines to CdA, Fara-A, and ara-C was determined (Fig. 1). The HL60/Fara-A mutant was two to five times more resistant to all analogues as compared with the parental cells, whereas the HL60/CdA cells showed several orders of magnitude higher resistance. Both resistant cell lines showed substantial cross-resistance to 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, difluorodeoxyguanosine, and difluorodeoxycytidine toxicity, only minor cross-resistance to 9- β -D-arabino-furanosylguanine and deoxyadenosine (with 10 μM EHNA), and no resistance to 5-fluorouracil (Table 1). The CdA-resistant cells also showed more than 4-fold cross-resistance to etoposide, a topoisomerase II inhibitor. Although the sensitivity for the ribonucleotide reductase inhibitor hydroxyurea was not much affected in HL60/CdA cells, the HL60/Fara-A cell line displayed more considerable resistance to this drug. The cytotoxicity of tubercidin, a marker of nucleoside transport deficiency, did not differ in the wild-type and the resistant cells (Table 1). Similarly, there was no difference in the toxicity of multidrug resistance-related drugs such as daunorubicin, vincristine, and paclitaxel between mutant and parental HL60 cells.

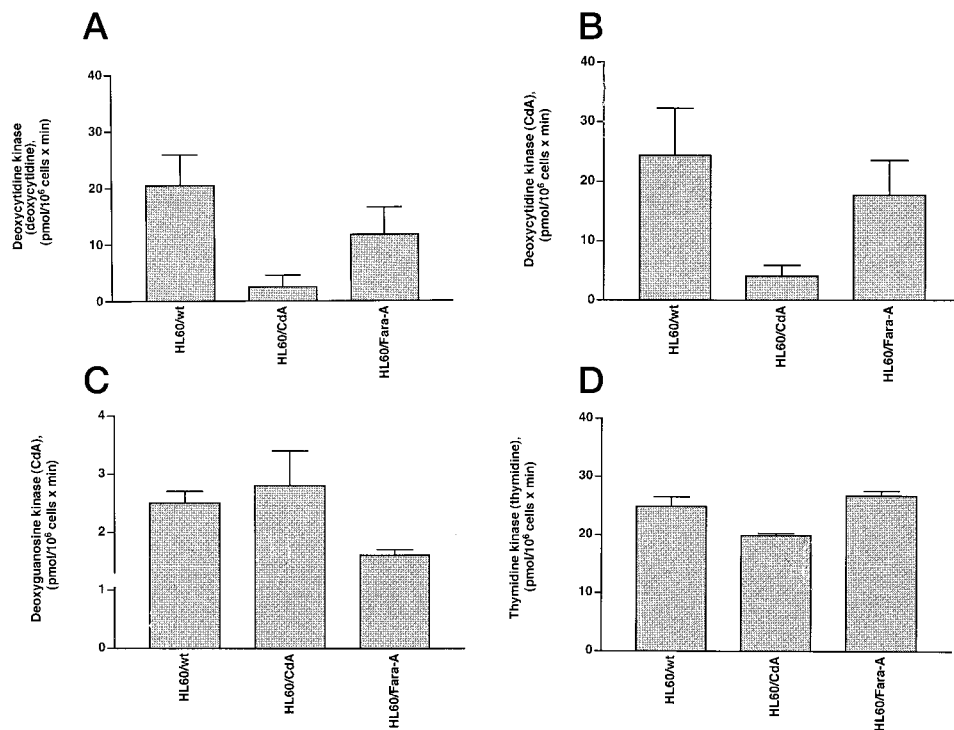
Characteristics of Resistant Cells. Table 2 shows a summary of the biological and biochemical characteristics of the resistant cell lines compared with HL60 wt cells. Neither the percentage of cells in G_1

Table 2 Biological and biochemical characterization of sensitive, CdA-, and Fara-A-resistant HL60 cells

	HL60/wt	HL60/CdA	HL60/Fara-A
Characterization			
Resistance factor	1	8244	5
% in S-phase	46	37	43
Doubling time (h)	34	35	29
Mono/Triphosphate formation ($\mu M/h$) after incubation with			
1 μM CdA	22.2/4.7	0.30/0.23	14.8/3.1
20 μM Fara-A	–/55.6	–/0.37	–/17.6
Uptake, DNA incorporation, and total phosphorylation (pmol/ 10^6 cells/h) of isotope analogues			
Thymidine			
Uptake	1.39 \pm 0.01 ^a	1.66 \pm 0.09	0.72 \pm 0.52
DNA incorporation	7.61 \pm 3.50	6.66 \pm 1.42	3.40 \pm 2.16
Total phosphorylation	8.99 \pm 3.50	8.32 \pm 1.51	4.11 \pm 2.67
Deoxycytidine			
Uptake	2.75 \pm 0.26	0.26 \pm 0.02	1.93 \pm 0.25
DNA incorporation	10.18 \pm 0.69	0.73 \pm 0.21	7.28 \pm 1.33
Total phosphorylation	12.93 \pm 0.95	0.99 \pm 0.23	9.21 \pm 1.59
CdA			
Uptake	19.70 \pm 7.32	4.10 \pm 3.26	12.76 \pm 2.45
DNA incorporation	3.67 \pm 0.93	2.30 \pm 0.85	3.42 \pm 0.80
Total phosphorylation	23.37 \pm 8.25	6.39 \pm 4.08	16.17 \pm 3.25
Fara-A			
Uptake	1.30 \pm 0.72	0.39 \pm 0.12	0.75 \pm 0.37
DNA incorporation	0.04 \pm 0.04	0.03 \pm 0.02	0.03 \pm 0.01
Total phosphorylation	1.34 \pm 0.76	0.42 \pm 0.14	0.78 \pm 0.39
Ara-C			
Uptake	4.24 \pm 1.06	0.17 \pm 0.09	2.68 \pm 1.76
DNA incorporation	0.14 \pm 0.06	0.01 \pm 0.00	0.12 \pm 0.07
Total phosphorylation	4.38 \pm 1.12	0.17 \pm 0.09	2.80 \pm 1.83

^a SD of at least three separate experiments in duplicate.

Fig. 2. Enzyme activities were determined in cell extracts from HL60/wt and resistant cells HL60/CdA and HL60/Fara-A with deoxycytidine (A), CdA (B), and thymidine (D). dGK activity was measured with CdA in the presence of 1 mM deoxycytidine (C). Cells were extracted immediately, and crude extracts were used for activity measurements as described in "Materials and Methods." Bars, SD.



and S phase, measured before and after four drug-free passages, nor the doubling times differed significantly.

Using HPLC the amounts of mono- and triphosphate of analogues were analyzed, showing that CdA-resistant cells had much lower levels of all phosphorylated metabolites. The amounts of CdA metabolites in Fara-A resistant cells were moderately decreased, whereas the Fara-ATP level was only 31% of those measured in parental cells (Table 2).

Uptake, Phosphorylation, and DNA Incorporation of the Analogues. DNA synthesis was measured using thymidine labeling of the cells, and the incorporation was found to be similar in the CdA-resistant cells and in the parental cells. However, the HL60/Fara-A cells showed significantly lower incorporation of thymidine into DNA, and the uptake and total phosphorylation of thymidine was also 50% of that observed in the control HL60 cells (Table 2). The most profound changes were observed in the metabolism of cytosine nucleosides in the HL60/CdA cells, where both uptake and incorpo-

ration of deoxycytidine and ara-C into DNA were markedly diminished. The total phosphorylation of purine nucleosides was also markedly reduced in this cell line, whereas the incorporation of purine nucleosides into DNA was much less affected. In HL60/Fara-A cells, both phosphorylation and incorporation of cytosine and purine nucleosides was reduced ~10–40% compared with parental cells (Table 2).

Determinations of dCK, dGK, and Thymidine Kinase Enzymes.

In vitro measurements of dCK with either deoxycytidine or CdA as substrate showed drastically diminished enzyme activity in HL60/CdA cell extracts, whereas the dCK level in extracts of HL60/Fara-A cells was only moderately reduced (Fig. 2, A and B). The activity of dGK, the mitochondrial purine phosphorylating enzyme that phosphorylates CdA and Fara-A, was not substantially decreased, and its relative contribution to the CdA phosphorylation was higher in the HL60/CdA cell extracts compared with the parental HL60 cells (Fig. 2C). The levels of thymidine kinase did not differ considerably in any cell line (Fig. 2D).

Western Blotting of dCK and dGK. The dCK and dGK protein levels in the HL60 cell lines were also determined using an antisera raised against a dCK peptide and a purified dGK antibody prepared against recombinant dGK (Fig. 3). The immunoassays showed that there was no detectable M_r 30,000 dCK protein in HL60/CdA extracts, whereas the dCK protein in extracts from HL60/Fara-A appeared to be at the same level as in the parental cells (Fig. 3A). The purified dGK antibody also cross-reacts with some proteins with higher molecular weight, but the band(s) corresponding to the size of the dGK protein were in all extracts at similar levels (Fig. 3B). The presence of two protein bands in the range of M_r 27,000–28,000 in the crude extracts may be due to the processing of dGK. The results of the immunological determinations are mainly in agreement with the enzyme assay, but in the immunoassays the problems arising from the overlapping substrate specificity of dCK, thymidine kinase 2, and dGK could be eliminated. However, the enhanced chemiluminescence immunodetection method used here is only a semiquantitative method and does not allow precise quantitative measurements. Thus, the

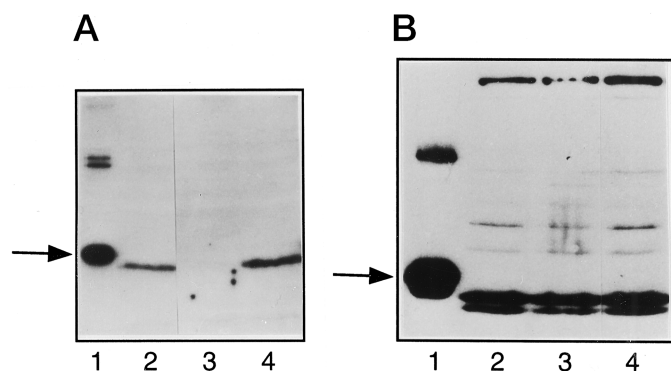


Fig. 3. SDS-PAGE and subsequent immunoblotting of dCK (A) and dGK (B) analyses with the purified recombinant dCK and dGK (Lane 1), cell line HL60 (Lane 2), and its CdA (Lane 3) and Fara-A (Lane 4) resistant sublines. The amount of crude protein applied on the gel was 40 μ g/lane, and the amount of recombinant dCK and dGK was 2 μ g. The recombinant enzymes contained a histidine tag at the NH₂-terminal, which adds M_r 2000–3000 to the respective enzyme.

Table 3 mRNA expression of dCK and 5'-NT in HL60 cells, using real-time quantitative PCR

	HL60/wt	HL60/CdA	HL60/Fara-A
dCK	0.559 ^a ± 0.080 (100 ^b)	0.052 ± 0.006 (9)	0.399 ± 0.012 (67)
5'-NT	0.101 ± 0.037 (100)	0.105 ± 0.019 (104)	0.115 ± 0.009 (114)
Ratio of dCK to 5'-NT	100 ^c	9	59

^a Ratio of dCK or 5'-NT and the internal control, β -microglobulin.

^b Percentage of wild-type (HL60/wt).

^c The ratio between dCK and 5'-NT, multiplied by 100.

decrease in dCK activity and in dCK mRNA expression (Table 3) could not be detected by Western blotting.

Deoxynucleotide Triphosphate Measurements. The intracellular levels of deoxynucleotide triphosphates were quantitated in HL60 cells by a DNA-polymerase assay. The pools varied in different experiments, depending on culture medium and conditions, but a trend was that the Fara-A-resistant cells contained significantly higher levels dATP, dGTP, and TTP pools compared with the parental cells (Fig. 4A–D). In the CdA-resistant cells, the levels of the dATP and TTP pools were approximately the same as in the parental cell line, and the dCTP pools were significantly lower in both resistant cell lines compared with wild-type cells (Fig. 4A–D). The overall results of pool measurement from DNA-polymerase assay were in agreement with results obtained by HPLC (data not shown).

Reverse Transcription-PCR and Real-time Quantitative PCR. Reverse transcription of total RNA from the CdA- and Fara-A-resistant HL60 cells and subsequent amplification of cDNA resulted in 783- and 1059-bp fragments for dCK and 5'-NT, respectively. This showed a normal length of DNA product for both mutants as compared with HL60/wt (data not shown). Using real-time quantitative PCR with β 2-microglobulin as internal standard, the expression of dCK mRNA showed similar results as the enzyme activity measurements and Western blot analysis. Although CdA-resistant cells have only 9%, the Fara-A resistant cells have 67% of dCK mRNA compared with parental cells (Table 3). mRNA expression of the cytosolic

enzyme 5'-NT was almost normal in HL60/CdA cells and slightly increased in the HL60/Fara-A cell line (104 and 114% of activity in parental cells, respectively).

DISCUSSION

In this study, we have developed resistant cell lines to nucleoside analogues by continuous exposure to CdA and Fara-A, which are frequently used in the treatment of hematological malignancies. Previously, it has been reported that chronic lymphocytic leukemia patients refractory to Fara-A responded to CdA (7), suggesting the absence of cross-resistance between these two analogues, and there is obviously a need to further delineate the mechanism of cross-resistance for these and other anticancer drugs.

We have shown in the present study that HL60/CdA cells are deficient in dCK, the enzyme required for activation of many cytosine-based and purine-based nucleoside analogues. These data are consistent with both previous findings in CCRF-CEM cells, in WIL2 human B lymphoblastoids, and in L1210 murine leukemia cell lines (21, 22).

The HL60/CdA mutants were >8000 times less sensitive to CdA, compared with HL60 wt (Fig. 1). No differences were detected regarding the cytotoxicity of tubercidin, indicating that transports of nucleosides was not responsible for CdA and Fara-A resistance in mutant HL60 cells. However, severe loss of sensitivity against

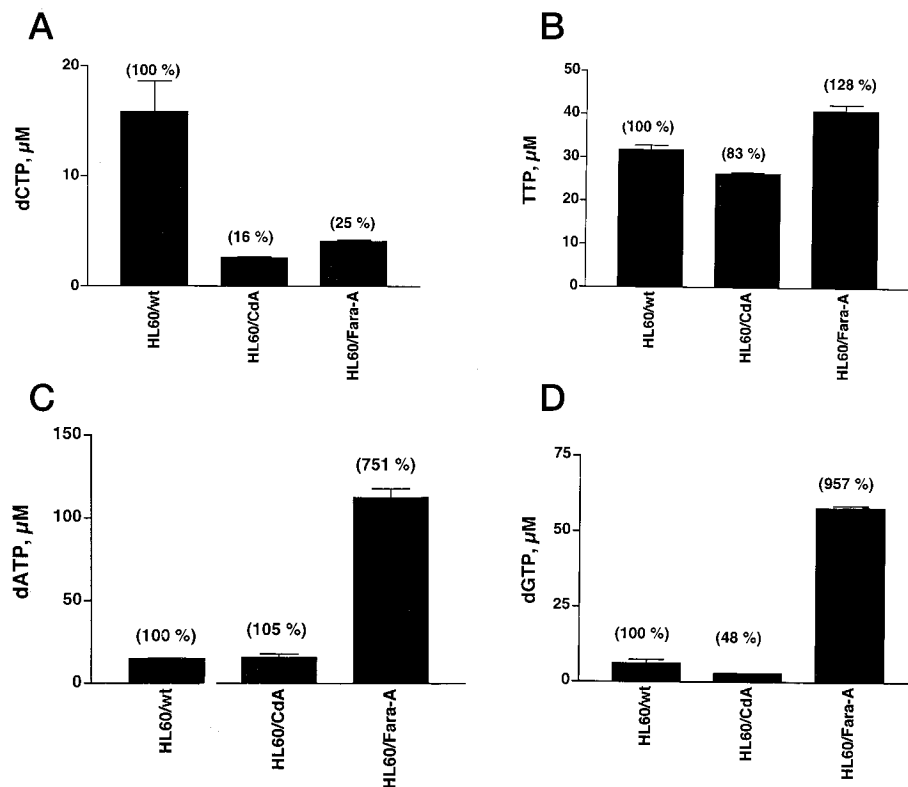


Fig. 4. The level of deoxyribonucleotide pools in wild-type and resistant HL60 cells. The quantitation of pools were done by DNA-polymerase assay as described in "Materials and Methods." HL60 cells were subcultured in RPMI 1640. Cells were harvested during logarithmic phase and washed with PBS, and $1-2 \times 10^6$ of cells were extracted by methanol. The data are means ($n = 4$); bars, SD. The data in parentheses show the percentages of respective nucleotide pools compared with wild-type (HL60/wt).

2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, Fara-A, ara-C, difluorodeoxycytidine, and difluorodeoxyguanosine was observed (Table 1). All of these nucleosides are activated mainly by dCK in lymphoid cells; thus, loss of dCK was expected to be the main reason for resistance in the HL60/CdA cell line. This was proved *in vitro* by measurements of dCK activity (Fig. 2, A and B) with deoxycytidine and CdA as substrates, as well as by the immunoblotting analysis where no dCK protein could be detected (Fig. 3A). The cellular resistance of HL60/CdA cells to several analogues is thus attributable to the deficiency of cytoplasmic dCK; similar mutant cells with defective dCK have been characterized in earlier studies (37–40).

Although neither thymidine kinase activity (Fig. 2) nor thymidine phosphorylation or incorporation into DNA were affected in HL60/CdA, phosphorylation of the deoxycytidine and purine nucleoside analogues was reduced compared with parental cells (Table 2). These data correlate well with *in vitro* dCK activity measurements, showing deficiency of dCK in HL60/CdA cells. The remainder phosphorylation of deoxycytidine and CdA with HL60/CdA cells and cell extracts is most likely attributable to the mitochondrial enzymes thymidine kinase 2 and dGK, respectively. In cells and tissues lacking dCK, the mitochondrial dGK enzyme is most likely responsible for the phosphorylation of CdA and Fara-A (41), and selection for high level resistance to these analogues could be expected to lead to alterations in the dGK levels. However, in the present study, we found no evidence for such a phenomenon, because the dGK levels were not changed. Surprisingly, the incorporation of purine nucleosides into DNA was hardly diminished in mutants compared with HL60/wt; this finding implicates that incorporation of CdA and Fara-A into DNA is not the mechanism of resistance.

Recently, a CdA-resistant HL60 cell line with higher expression of 5'-NT mRNA and higher activity of the cytosolic enzyme has been described (24). These cultured, resistant cells showed no cross-resistance to ara-C and difluorodeoxycytidine and showed only slightly reduced dCK activity, but the ratio of dCK to 5'-NT was reduced for the mutants compared with parental cells. In contrast, the HL60/CdA mutant developed in our study showed drastically decreased dCK activity but had an unchanged 5'-NT mRNA expression. One of the reasons for this discrepancy could be the way cells were adapted to the drugs.

The mechanism of dCK deficiency is mainly unknown. One possible mechanism could be the DNA methylation that regulates gene expressions in mammalian cells. However, no DNA methylation of the *dCK* gene was observed in CCRF-CEM cells resistant to CdA (42).

HL60/Fara-A cells showed a 40% decreased dCK level, and the accumulation of Fara-ATP in these cells was reduced to a similar extent, indicating that dCK is the rate-limiting step in the activation of Fara-A as demonstrated also in earlier studies (5). The decrease in dCK activity, as well as some discrepancy between activity measurements and Western blotting data, showing no decrease in dCK protein, may be explained by increase in 5'-NT (114% compared with wild type; Table 3). This cell line showed 5- and 2-fold cross-resistance to ara-C and difluorodeoxycytidine. However, the reduced phosphorylation is most likely not the only factor involved in the resistance, and the observed elevation of the intracellular deoxynucleoside triphosphate pools may also be a contributing factor. Recently, it has been shown that Fara-ATP can cooperate with Apaf-1 and cytochrome *c* to activate the caspase pathway more effectively than dATP (20). Excessive levels of dATP measured in Fara-A mutants may not only compete with Fara-ATP for incorporation into DNA but may also compete with Fara-ATP to cooperate with cytochrome *c* and Apaf-1 to induce apoptosis. Ribonucleotide reductase is allosterically inhibited by Fara-ATP, which leads to the reduction of the dCTP and dATP

pools in treated cells (43). It is thus likely that continuous exposure to Fara-A may select ribonucleotide reductase mutants with reduced sensitivity to the allosteric inhibitors. A severe decrease in dCTP level and the high levels of other DNA precursor pools in HL60/Fara-A (Fig. 4) is probably the result of a dominant mutation in the ribonucleotide reductase gene so that it is no longer as sensitive to allosteric feedback control as the parental cells. These types of mutants have been described earlier and defined in detail (44–48), and the cross-resistance to other toxic nucleoside analogues observed with HL60/Fara-A was also seen with these mutants. Additional studies in attempt to measure the allosteric control of the ribonucleotide reductase reaction in the resistant cell lines are now in progress.

The results presented here demonstrate that in HL60 cells selected for resistance to CdA and Fara-A, several different regulatory steps were altered, *i.e.*, the phosphorylation and dephosphorylation carried out by dCK and 5'-NT, respectively. The levels of the deoxynucleotides used for DNA synthesis were also altered, presumably because of alteration in ribonucleotide reductase enzyme. In the case of CdA resistance, the first process seems to be dominant because the highly resistant cells, showing cross resistance to several other nucleoside analogues, was found and was associated with dCK deficiency. However, nucleoside-resistant cells in the clinic were found only in a low frequency to be dCK deficient (48), and the reason for this discrepancy between cell culture results and the clinical resistant cells is not clear. The HL60/Fara-A cells appear to be primarily ribonucleotide reductase mutants, and similar types of cells have earlier been shown to have increased spontaneous mutation frequencies attributable to the altered DNA precursor level (45–47). Therefore, a low level resistance to toxic nucleosides, *e.g.*, Fara-A, could be attributable to a dominant ribonucleotide reductase mutation, and this may be an intermediate in the selection of more highly resistant cells as a result of the higher mutation frequency of this type of cells. Our results encourage additional studies to define the role of ribonucleotide reductase mutants as well as dCK mutations in clinically isolated, nucleoside-resistant tumor cells.

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