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Effect of 5-Azacytidine and Congeners on DNA Methylation and Expression of Deoxycytidine Kinase in the Human Lymphoid Cell Lines CCRF/CEM/0 and CCRF/CEM/dCk$^-$ [1]

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ABSTRACT

The human lymphoid cell lines CCRF/CEM/0 and the deoxycytidine kinase (dCk)-deficient CCRF/CEM/dCk$^-$ were treated with various 5-azacytidine (5-aza-C) nucleosides and the effect on DNA methylation and dCk activity were examined. 5-Azacytidine (5-aza-C), 5,6-dihydro-5-azacytidine (DHAC), 5-aza-2′-deoxycytidine (5-aza-Cdr), and 1-β-D-arabinofuranosyl-5-azacytidine (ara-AC) reduced the DNA 5-methylcytosine level in the CEM/0 cells, down to approximately 10% of the level in untreated cells. The dCk activity was increased after treatment with the 5-aza-C nucleosides approximately 10% compared to untreated cells. In CEM/dCk$^-$ cells DNA hypomethylation between 50 and 25% of control was seen only after treatment with DHAC and 5-aza-C. No decrease in the methylation level was seen after treatment with 5-aza-Cdr or ara-AC. The dCk activity was increased up to 37% after treatment with DHAC or 5-aza-C but no increase was observed after treatment with 5-aza-Cdr or ara-AC. CEM/dCk$^-$ cells treated with DHAC showed a revertant frequency to cells expressing dCk activity of between 0.1 and 0.6%. Cloned revertant CEM/dCk$^-$ cells isolated from soft agar had dCk activity between 31 and 113% compared to the activity in untreated CEM/0 cells. This *in vitro* study indicates that DHAC and 5-aza-C induced dCk re-expression in the CEM/dCk$^-$ cells whereas 5-aza-Cdr and ara-AC did not.

INTRODUCTION

The enzymatically mediated posttranscriptional transfer of a methyl group from *S*-adenosylmethionine to the 5-carbon atom of cytosine results in the formation of 5-methylcytosine, the only modified base known to occur in mammalian DNA (1, 2). Although the biological function of DNA methylation is imperfectly understood, methylation is considered to be one of several mechanisms involved in differentiation (3, 4) and regulation of gene transcription (5, 6).

Analyses using methylation-sensitive restriction enzymes have shown that many genes are hypomethylated in their 5′ flanking regions when being actively transcribed and methylated when in the nontranscribed chromatin conformation (7–9). For example the β-globin gene is methylated in cells not expressing the gene product whereas in cells expressing β-globin the gene is hypomethylated (7, 8). The hypoxanthine phosphoribosyltransferase gene has been found to have different methylation levels on the active and inactive X-chromosome, the gene on the active X-chromosome being relatively hypomethylated (9). Further evidence in support of an inverse relationship between DNA methylation and active gene transcription has come from studies utilizing a hypomethylating agent such as 5-aza-C[3] (10–12).

5-aza-C is a derivative of cytosine with a nitrogen in the 5 position of the pyrimidine ring (13). The nucleoside is phosphorylated in cells to the biologically active triphosphate and incorporated into the nucleic acids (14). When incorporated into DNA it induces hypomethylation (15). 5-aza-C is unstable in aqueous solution at neutral pH and decomposes to a compound of unknown biological activity (16). DHAC is a derivative of 5-aza-C with pharmacological activity and extensive stability in aqueous solution (17, 18).

ara-C is a nucleoside derivative which has received considerable attention as a result of its clinical efficacy against a spectrum of human malignancies (19–21). However, its therapeutic usefulness is hindered by the frequent development of resistance in tumor cell populations after repeated ara-C exposure (22, 23). The resistance is often associated with a decrease in dCk activity. *In vitro* work by several authors has shown that ara-C is capable of inducing enzymatic hypermethylation of DNA (24, 25). It therefore becomes an attractive hypothesis that resistance to ara-C is related to hypermethylation at the dCk gene locus, occurring as a consequence of inhibition of DNA synthesis during therapy. If so, resistance to ara-C might be reversible by exposure of resistant tumor cell populations to hypomethylating agents. Indeed, preliminary results from our laboratory have suggested that 5-aza-C treatment is capable of re-establishing sensitivity toward ara-C in pediatric patients resistant to this drug (26–28).

The present study was undertaken to define on the biochemical level the mechanisms by which 5-aza-C and its congeners re-establish ara-C sensitivity in resistant human tumor cells. To this end, the effects of treatment with 5-carbon-substituted cytidine analogues upon DNA methylation and dCk re-expression were examined in a dCk-deficient human leukemia cell line.

MATERIALS AND METHODS

RPMI 1640 medium and heat-inactivated fetal calf serum were from Irvine Scientific, Irvine, CA, [6-^{3}H]uridine and [5,6-^{3}H]ara-C were from Moravek Biochemicals Inc., Brea, CA. DE 81 filter paper discs were from Whatman, ara-C was from the Upjohn Co., and 5-aza-C and 5-aza-Cdr were purchased from Sigma. DHAC and ara-AC were kindly provided by the Drug Development Branch, National Cancer Institute, NIH. The CCRF/CEM/0 and CCRF/CEM/dCk$^-$ cells were generous gifts from Dr. Arnold Fridland, St. Jude's Children's Research Hospital, Memphis, TN. All other chemicals were of analytical or HPLC quality.

Radioactive ara-C "Substrate Mixture". The [5,6-^{3}H]ara-C was purified by passage through a DEAE-Sepharose column equilibrated in

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[3] The abbreviations used are: 5-aza-C, 5-azacytidine; DHAC, 5,6-dihydro-5-azacytidine; ara-AC, 1-β-arabinofuranosyl-5-azacytidine; 5-aza-Cdr, 5-aza-2′-deoxycytidine; ara-C, 1-β-arabinofuranosylcytosine; 5-mC, 5-methylcytosine; dCk, deoxycytidine kinase; PBS, phosphate-buffered saline; HPLC, high-performance

water. This purification step decreased the background in the filter assay about 5 times. The Substrate Mixture contained 250 μM [^{3}H]ara-C in buffer A (50 mM Tris-HCl, 20 mM $MgCl_2$, 10 mM KF, 20 mM ATP, 0.10 mM tetrahydrouridine, pH 7.6); the specific activity was 120 dpm/pmol. The counting efficiency was 50 ± 2%.

Culture Condition. The human leukemia cell lines CCRF/CEM/0 and CCRF/CEM/dCk⁻ were grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum and 10 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid, pH 7.2. The cell cultures were incubated at 37°C in a humidified air atmosphere containing 5% CO_2 (NAPCO Model 6100) and were subcultured every 3–4 days keeping the cell density between 1×10^5 and 1×10^6 cells/ml.

Determination of the IC_{50} Concentrations. The cells were suspended in culture medium at a density of 3×10^5 cells/ml and incubated with increasing concentrations of the nucleosides over a range of 3 logs. After a 24-h incubation at 37°C the cultures were washed twice with culture medium and resuspended in drug-free medium to the original volume. After 24, 48, and 72 h the cells were counted and the nucleoside concentration causing 50% of control growth inhibition was determined.

Treatments with Nucleosides. The cells were suspended in culture medium at a density of 3×10^5 cells/ml. The various nucleosides, dissolved in PBS, were added to desired concentrations and the cultures were incubated at 37°C for 24 h. At the end of the incubation period the cells were collected by centrifugation, washed twice with culture medium, and resuspended in culture medium to the original volume. At various time intervals during or after the incubation, samples were withdrawn for determination of DNA methylation and dCk activity.

Determination of DNA Methylation Level. In the presence of 10 μCi [6-^{3}H]uridine 3×10^5 cells were incubated in 2 ml of culture medium at 37°C for 24 h. At the end of the incubation period the cells were washed once with PBS and lysed by incubation in 0.3 M NaOH, containing 0.1% sodium dodecyl sulfate, at 37°C for 24 h. During this incubation the RNA was hydrolyzed. After adjustment of pH to 7.6 the samples were incubated with proteinase K (100 μg/ml) at 65°C for one h. After cooling the samples to 4°C the DNA was precipitated by addition of 50% trichloroacetic acid to a final concentration of 10%. The precipitate was washed consecutively with 5% ice-cold trichloroacetic acid and 70% ice-cold ethanol and finally dried. The purified DNA was hydrolyzed to its bases in 88% formic acid at 180°C for 50 min. After drying and reconstitution with PBS the bases were separated isocratically by HPLC (Waters) on an SCX column at room temperature in 60 mM phosphate buffer, pH 2.5, at a constant flow rate of 0.6 ml/min (Fig. 1). The eluate was monitored at 280 nm and collected in fractions of 0.6 ml. The fractions were measured for radioactivity by scintillation counting. The amount of radioactivity eluted with cytosine (t_R 9 min) and 5-mC (t_R 17 min) were determined and the percentage of 5-mC was calculated according to the following formula.

$$\text{5-mC} = \frac{\text{cpm 5-mC} \times 100}{\text{cpm 5-mC} + \text{cpm cytosine}}$$

Assay of Deoxycytidine Kinase Activity. Cells (2×10^7) were collected by centrifugation and washed once with PBS. The washed cells were resuspended in 200 μl buffer B (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.6) and the cells were lysed by sonication for 30 s. Cell debris was removed by centrifugation at 15,000 rpm for 1 h in a Sorvall RC2-B refrigerated centrifuge using a SS-34 rotor. The supernatant was collected and protein concentration was determined spectrophotometrically according to the method of Ehresmann *et al.* (29). The samples were diluted with buffer B to a protein concentration of 0.6 mg/ml for the CEM/0 cells and to between 10 and 15 mg/ml for the CEM/dCk⁻ cells. After preheating of the sample dilutions and the [^{3}H]ara-C Substrate Mixture at 37°C for 10 min 20 μl of sample dilution were mixed with 20 μl Substrate Mixture and further incubated at 37°C for between 15 min and 2 h (30). At the end of the incubation period the reaction was quenched by addition of 10 μl of 0.2 M EDTA solution.

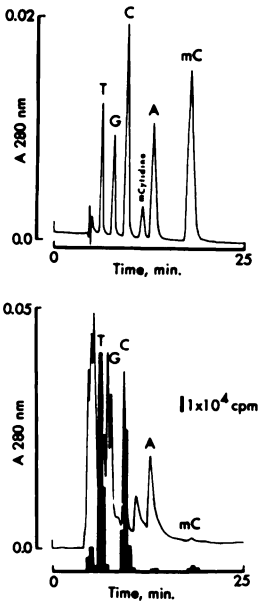

Fig. 1. HPLC chromatogram of nucleoside bases. The bases were separated isocratically on an SCX column at room temperature in 60 mM phosphate buffer, pH 2.5, at a constant flow rate of 0.6 ml/min. The eluate was monitored at 280 nm and collected in fractions of 0.6 ml. *Upper part*, separation of a standard solution; *lower part*, separation of hydrolyzed DNA from CEM/0 cells after incubation with [6-^{3}H]uridine for 24 h. *Bars*, distribution of the radioactivity. *T, G, C, A*, and *mC*, thymidine, guanine, cytosine, adenine, and 5-mC, respectively.

allowed to dry for 10 min and then immersed into and washed 5 times in deionized water. After the washing procedure the discs were transferred to scintillation vials, 1 ml of a solution of 0.2 M KCl/0.2 M HCl was added, and the samples were incubated for 30 min. Finally 10 ml of scintillation fluid was added and the radioactivity was measured by scintillation counting. The dCk activity was calculated and expressed as pmol phosphorylated ara-C/mg protein × min.

***In Vitro* Cloning in Selective Agar of dCk Expressing Cells.** The cells were treated with 0, 20, or 100 μM DHAC for 24 h, washed with PBS, and grown in drug-free medium for 72 h prior to plating. The cells were plated in 60-mm Petri dishes in RPMI 1640 medium containing: 10% heat inactivated calf serum; agar; 30, 60, or 100 μM thymidine; 10 or 30 μM deoxycytidine; combinations of thymidine and deoxycytidine or no nucleosides. The dishes were prepared with two agar layers; the lower layer contained 3 ml medium with 0.5% sea plaque agar, and the upper layer contained the cells in 2 ml medium with 0.25% agar. Preliminary results showed plating efficiencies for CEM/0 cells of 35 to 100% in medium with deoxycytidine or no added nucleosides and less than 9 and 5% in 30 and 60 μM thymidine, respectively. The cells were completely rescued from the thymidine effect by 10 or 30 μM deoxycytidine. The CEM/dCk⁻ cells had similar plating efficiencies of 35 to 100% in deoxycytidine or without nucleosides. However, they were much more sensitive to thymidine and were not rescued by deoxycytidine. The number of plated cells per dish varied from 200 to 5×10^4, depending on the medium, to optimize sensitivity and counting of colonies. Several thymidine concentrations were used to ensure that suppression of growth would be sufficient to detect revertant colonies only. After 14 to 21 days incubation at 37°C the colonies on the various dishes were counted, 5 to 10 dishes were counted for each condition, and the revertant frequency was calculated according to the following equation

$$\text{Revertant frequency} = TC/C - T/N$$

where TC, C, T, and N were the plating efficiencies in the agar plates containing thymidine plus deoxycytidine, deoxycytidine alone, thymidine alone, and no nucleoside, respectively.

As a control, 0.1 to 5% CEM/0 cells were mixed with CEM/dCk⁻ cells and between 4.5×10^3 and 5.8×10^4 of these cells were plated in

agar plates was seen. This shows that revertant cells can be detected and that the large number of CEM/dCk⁻ cells do not interfere with the plating efficiency of CEM/0 cells.

Colonies were removed from the thymidine plus deoxycytidine agar plates and were grown up in suspension. In order to determine the amount of dCk in the clones the following assay was performed. Cells (1×10^7) were suspended in 2 ml culture medium and incubated in the presence of 200 μM ara-C for 1 h at 37°C. At the end of the incubation period the cells were extracted with perchloric acid and the nucleotide containing acid soluble fraction was analyzed by HPLC. Separation of the nucleosides was performed at room temperature on a SAX column developed with a linear gradient of 0.05 M $NH_4H_2PO_4$, pH 2.8, to 0.75 M $NH_4H_2PO_4$, pH 3.5, at a constant flow rate of 2.0 ml/min. The eluate was monitored at 280 nm and the amount of formed ara-CTP was calculated from the integration area of the elution peak corresponding to ara-CTP.

RESULTS

The effect of DHAC and the other 5-aza-C derivatives on DNA methylation and dCk expression was examined. Changes in DNA methylation level after exposure to the nucleosides were compared to dCk activity.

IC_{50} Concentrations. The IC_{50} concentrations of the 5-aza-C nucleosides for the CEM/0 and CEM/dCk⁻ cells are shown in Tables 1 and 2. The IC_{50} concentrations for the CEM/0 cells were between 0.3 and 100 μM for the different nucleosides, ara-AC being the most and DHAC the least toxic. For CEM/dCk⁻ cells the IC_{50} concentrations were 200 μM for DHAC and 40 μM for 5-aza-C. 5-aza-Cdr and ara-AC showed very low toxicity on the CEM/dCk⁻ cells with IC_{50} concentrations of 500 μM and over 1 mM, respectively.

DNA Methylation. The methylation levels in CEM/0 and CEM/dCk⁻ cells at various time points after a 24-h treatment with DHAC are shown in Fig. 2. The methylation levels in untreated cells were for CEM/0, 3.72 ± 0.07% 5-mC, and for CEM/dCk⁻, 3.06 ± 0.09% 5-mC. The nadir in methylation level was seen in both cell lines 24 h after the end of the exposure to DHAC. In the CEM/0 cells the methylation level decreased to 1.49% 5-mC or 40% of the methylation level in

Table 1 *DNA methylation levels in CEM/0 cells at different time points after a 24-h treatment with various 5-aza-C nucleosides*

The methylation level in untreated CEM/0 cells was 3.72 ± 0.07%. Percentage of 5-mC and methylation levels expressed as percentage of control, *i.e.*, 3.72% = 100%, are given for various time points. Each data point is the average of at least 3 determinations ± SD.

Nucleoside	Concentration[a] (μM)	Methylation levels at various time points from beginning of treatment: 24 h (% 5-mC)	%	48 h (% 5-mC)	%	79 h (% 5-mC)	%
DHAC	1			3.83 ± 0.06	103		
	10			3.06 ± 0.13	82		
	20			2.99 ± 0.02	80	3.60 ± 0.13	97
	100	2.23 ± 0.28	60	1.49 ± 0.28	40	2.75 ± 0.12	74
	200	1.73 ± 0.23	47	0.72 ± 0.20	19	1.99 ± 0.06	53
5-aza-C	1	2.29 ± 0.23	62	3.21 ± 0.08	86		
	3	1.24 ± 0.13	33	2.22 ± 0.04	60		
	6	1.20 ± 0.20	32	1.32 ± 0.02	35	2.92 ± 0.06	78
	10	0.78 ± 0.08	21				
5-aza-Cdr	0.1	2.63 ± 0.13	71				
	0.3	1.75 ± 0.21	47				
	0.5	0.40 ± 0.05	11	1.99 ± 0.07	53		
	4	0.26 ± 0.04	7[b]	0.41 ± 0.06	11	1.37 ± 0.02	37
ara-AC	0.5	1.06 ± 0.04	28	2.84 ± 0.06	76		
	3	0.97 ± 0.37	26	0.96 ± 0.02	26	2.63 ± 0.04	71
	5			0.96 ± 0.02	26		
ara-C	15			3.82 ± 0.06	103		

[a] IC_{50} values were 100, 3, 1, and 0.25 μM for DHAC, 5-aza-C, 5-aza-Cdr, and ara-AC, respectively.
[b] 5-mC cpm were 7-fold higher than background.

Table 2 *DNA methylation levels in CEM/dCk⁻ cells at different time points after a 24-h treatment with various 5-aza-C nucleosides*

The methylation level in untreated CEM/dCk⁻ cells was 3.06 ± 0.09%. Percentage of 5-mC and methylation levels expressed as percentage of control, *i.e.*, 3.06% 5-mC = 100%, are given for various time points. Each data point is the average of at least 3 determinations ± SD.

Nucleoside	Concentration[a] (μM)	Methylation levels at various time points from beginning of treatment: 24 h (% 5-mC)	%	48 h (% 5-mC)	%	79 h (% 5-mC)	%
DHAC	10	3.09 ± 0.08	101	2.77 ± 0.08	91		
	50			2.27 ± 0.31	74		
	100	2.05 ± 0.03	67	1.94 ± 0.13	63	2.55 ± 0.06	83
	200	1.65 ± 0.10	54	1.40 ± 0.10	46	2.44 ± 0.12	80
	500	1.48 ± 0.04	47	1.37 ± 0.18	45	2.35 ± 0.11	77
	1000	1.28 ± 0.16	42	1.39 ± 0.23	45	2.45 ± 0.25	80
5-aza-C	1	2.93 ± 0.04	96	2.94 ± 0.08	96		
	50	0.56 ± 0.19	18	0.72 ± 0.12	24	2.17 ± 0.10	71
5-aza-Cdr	5	3.02 ± 0.10	99	3.00 ± 0.04	98		
	360			2.84 ± 0.18	93		
ara-AC	5	3.16 ± 0.06	103	3.01 ± 0.27	98		
	1000	3.04 ± 0.09	99	3.06 ± 0.12	100	3.12 ± 0.06	102
ara-C	150	3.14 ± 0.10	103	3.06 ± 0.10	100		
	500			3.45 ± 0.04	113		

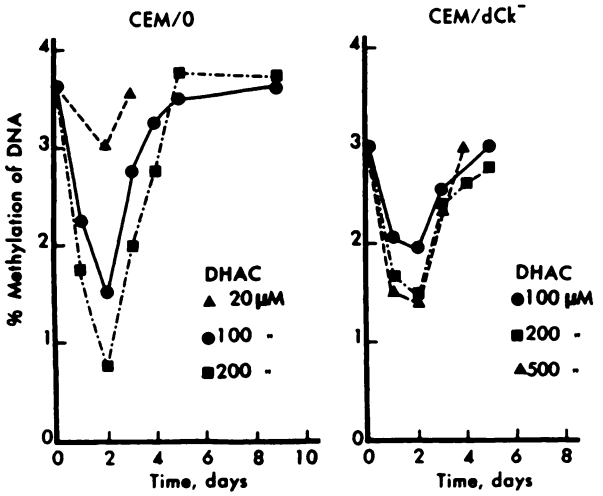

Fig. 2. DNA methylation levels at 24-h intervals in CEM/0 and CEM/dCk⁻ cells from the beginning of a 24-h treatment with various DHAC concentrations. The methylation levels in untreated cells were 3.72 ± 0.07% and 3.06 ± 0.09% in CEM/0 and CEM/dCk⁻ cells, respectively.

untreated cells after treatment with 100 μM DHAC and to 0.72% 5-mC or 19% of control after treatment with 200 μM DHAC. With 1 μM DHAC no effect of the methylation level was seen in the CEM/0 cells; 10 μM DHAC decreased the methylation level to 82% of the level in untreated cells (Table 1). Treatment of CEM/dCk⁻ cells with 100 μM DHAC decreased the methylation level to 1.94% 5-mC or 63% of the level in untreated cells; 200 μM DHAC gave a methylation level of 1.40% 5-mC or 46% compared to untreated cells. DHAC concentrations of 500 μM or 1 mM did not significantly further decrease the methylation level. However, the induced hypomethylation was of transient nature in both cell lines and the methylation levels had returned by day 7 almost to control levels (Fig. 2).

The hypomethylating effect of DHAC was not increased in either of the two cell lines by repeated exposures to the nucleoside. The cells were treated with DHAC every fifth day for 24 h and the methylation levels were determined 24 h after the end of each treatment. The methylation levels after the first exposure to DHAC were the lowest levels obtained; additional treatments could not fully achieve the same extent of hypomethylation. After the first exposure to 100 and 200 μM DHAC the methylation level decreased to 1.49 ± 0.28% and 0.72 ± 0.20% in the CEM/0 cells and to 1.94 ± 0.13% and 1.40 ± 0.10% in the CEM/dCk⁻ cells, respectively. However, after the third exposure the methylation level was 1.98 ± 0.04% and 1.30 ± 0.11% in the CEM/0 cells and 2.28 ± 0.05% and 1.82 ± 0.17% in the CEM/dCk⁻ cells, respectively.

The effect of the other 5-aza-C nucleosides on the DNA methylation levels in the two cell lines are presented in Tables 1 and 2. All nucleosides caused DNA hypomethylation in the CEM/0 cells whereas in the CEM/dCk⁻ cells hypomethylation was seen after treatment with DHAC and 5-aza-C only. In CEM/0 cells 5-aza-Cdr was the most potent hypomethylating agent. Half the IC_{50} concentration, 0.5 μM, decreased the methylation level to 0.40% 5-mC or 11% of the methylation level in untreated CEM/0 cells; this is an exceptionally low level of 5-mC. Treatment of CEM/dCk⁻ cells with 5-aza-Cdr or ara-AC did not have any effect on the methylation level even at 1 mM (Table 2). The nadir in methylation level for DHAC was seen 24 h after the end of exposure to the nucleoside whereas for Treatment with ara-C produced no change in the methylation levels (Tables 1 and 2).

Deoxycytidine Kinase Expression. The dCk activity in the cells before and after treatment with the various 5-aza-C nucleosides was measured as described under "Materials and Methods." In untreated CEM/0 cells the dCk activity was 102 pmol/mg × min. This activity was increased after treatment with all 5-aza-C nucleosides. The enzyme activity 48 h from the beginning of the 24-h treatments was increased about 10% compared to untreated cells; 48 h later the activity had returned to control level. No significant difference between the nucleosides could be detected. Treatment with 100 μM DHAC increased the activity to 112 ± 9 pmol/mg × min or to 110% of control. Increasing the concentration of DHAC from 100 to 200 μM did not enhance the dCk activity. 5-aza-C, 5-aza-Cdr, and ara-AC increased the activity to 111, 106, and 111%, respectively, compared to the activity in untreated CEM/0 cells. CEM/0 cells treated with ara-C showed a decrease in dCk activity to about 70% of the activity found in untreated cells.

The CEM/dCk⁻ cell line is deficient in dCk and untreated cells had a dCk activity of between 0.3 and 0.7 pmol/mg × min or less than 1% of the activity in CEM/0 cells. At this very low level of enzyme activity the variation between assays on different days was somewhat larger than the variation within an experiment. The reproducibility within an experiment had standard deviations of 0.01 to 0.05 pmol/mg × min.

After treatment with DHAC the dCk activity increased up to 26% compared to the activity in untreated CEM/dCk⁻ cells (Table 3). Treatment with 100 μM DHAC increased the dCk activity about 16% and treatment with 500 μM or 1 mM about 25%, showing a dose-response relationship. Treatment with 50 μM 5-aza-C increased the dCk activity approximately 35% compared to untreated cells. On the other hand, treatment with 5-aza-Cdr or ara-AC did not increase the dCk activity in the CEM/dCk⁻ cells. ara-C had no effect on the dCk activity (Table 3). Repeated treatments of the CEM/dCk⁻ cells with DHAC did not increase dCk activity compared to a single treatment.

After treatment with 100 and 200 μM DHAC CEM/dCk⁻ cells were grown in suspension and at various time points samples were withdrawn and assayed for dCk activity. As shown in Table 3 the increase in enzyme activity was persistent and essentially constant over at least a period of 8 days.

***In vitro* Cloning in Selective Agar of dCk Expressing Cells.** Three separate experiments were performed with CEM/0 and CEM/dCk⁻ cells. Considerable variation was seen among the three experiments. Nevertheless, in all three experiments CEM/

Table 3 *Deoxycytidine kinase activity in CEM/dCk⁻ cells after treatment with the various 5-aza-C nucleosides*

The cells were treated with the nucleosides for 24 h and the enzyme activity was determined at various time points after the treatments. Each data point is the average of at least 5 determinations ± SD.

Nucleoside concentration	dCk activity at various time points from beginning of treatment					
	48 h dCk[a]	%[b]	96 h dCk[a]	%[b]	192 h dCk[a]	%[b]
Control	0.73 ± 0.06	100	0.66 ± 0.02	100	0.66 ± 0.05	100
DHAC 100 μM	0.85 ± 0.02	116	0.71 ± 0.02	108	0.76 ± 0.05	115
DHAC 200 μM	0.86 ± 0.03	117	0.75 ± 0.03	114	0.78 ± 0.08	117
DHAC 500 μM	0.92 ± 0.03	126				
DHAC 1 mM	0.88 ± 0.04	121				
5-aza-C 50 μM	1.00 ± 0.14	137				
5-aza-Cdr 360 μM	0.66 ± 0.13	90				
ara-AC 1 mM	0.73 ± 0.02	100				
ara-C 150 μM	0.78 ± 0.04	106				
ara-C 500 μM	0.71 ± 0.06	97				

Table 4 *Revertant frequency of CEM/dCk⁻ cells after treatment with DHAC*

The cells were treated with DHAC for 24 h, grown in suspension for 72 h, and plated in for dCk expression selective soft agar plates. After 14 to 21 days of incubation the colonies on the plates were counted and the revertant frequency was calculated.

Experiment	Treatment DHAC (μM)	Revertant frequency[a] × 10^{-5}
1	0[b]	0
	100	61
	0[b]	160
	100	585
2	0	0
	20	1
	100	81
3	20	290
	100	360

[a] Reversion frequency calculated according to text.
[b] Two different CEM/dCk⁻ lines were used in the experiment.

Table 5 *Deoxycytidine kinase activity measured as ara-CTP formation in CEM/0 and CEM/dCk⁻ clones isolated from soft agar plates selective for cells expressing dCk*

The clones were removed from the agar plates, grown up in solution, and assayed for ara-CTP formation.

Cells	Formation of ara-CTP (nmol of ara-CTP 1 × 10^{-7} cells)	% of CEM/0 control
CEM/0		
Control	1.12	100
Clone	1.20	102
Clone	0.85	68
CEM/dCk⁻		
Control	0.02[a]	1.8
Control	0.08[a]	6.9
Control	0.04[a]	3.8
Revertant	1.10	93
Revertant	0.58	49
Revertant	1.27	113
Revertant	1.08	97
Revertant	0.35	31

[a] Quantitated manually.

dCk⁻ cells treated with DHAC gave more colonies on the agar plates with combinations of thymidine and deoxycytidine compared to untreated controls or the treated cells grown in thymidine only. Table 4 shows the revertant frequencies, calculated as described under "Materials and Methods," for the three experiments. The values of 0 to 0.2% seen in untreated cells probably indicate a fraction of revertant cells in the CEM/dCk⁻ cells at the time of the experiment. DHAC treatment increased the revertant frequency up to 0.6%. The treated CEM/0 cells grew normally in the thymidine plus deoxycytidine agar.

Ten colonies of CEM/dCk⁻ revertants from DHAC treatments were removed from agar plates containing thymidine and deoxycytidine and individually grown up in suspension. The dCk activity in these clones was determined from their ability to phosphorylate ara-C to ara-CTP (27, 28). The clones of treated CEM/dCk⁻ cells isolated from the selective agar plates formed between 31 to 113% ara-CTP compared to untreated CEM/0 cells (Table 5). This activity was 8- and 27-fold higher than in the untreated CEM/dCk⁻ cells plucked from control agar plates.

DISCUSSION

The problem of acquired drug resistance has been called perhaps the most important problem currently facing research-populations of cells during tumor progression would occur by mutational mechanisms. Recent advances in our understanding of the control of eukaryotic gene expression, however, suggest that epigenetic mechanisms may also play an important role in the diversification of tumor cell populations during tumor progression (32, 33). Among these epigenetic phenomena may be the loss of stable patterns of DNA methylation (34).

The CEM/dCk⁻ cells have been made resistant to ara-C by repeated exposures to the nucleoside. The CEM/dCk⁻ cells are deficient in dCk, the enzyme required for phosphorylation of deoxy- and arabinosylcytosine derivatives (35). Other ara-C-resistant cells have also shown deficiency in dCk activity (36, 37), which would indicate that loss of dCk activity is a common mechanism of resistance to this nucleoside drug. However, total lack of dCk activity has not been observed in clinically resistant cell populations isolated from patients (38, 39).

We have shown in the present study that the DNA hypomethylating agents 5-aza-C and DHAC induce a genome-wide hypomethylation in both CEM/0 lymphoid cells and their CEM/dCk⁻ mutants. Consistent with our previous finding that 5-aza-Cdr and ara-AC are not phosphorylated to their triphosphates or incorporated into DNA of CEM/dCk⁻ cells, we found these agents induced hypomethylation only in CEM/0 cells (40).

Treatment periods of 24 h, corresponding to approximately one cell cycle for CEM cells, ensured S-phase exposure of the total cell population to 5-aza-C and its tested derivatives (41). While maximum hypomethylation following DHAC treatment occurred 24 h after cessation of treatment, it occurred immediately at the end of treatment for 5-aza-C and its other derivatives. This difference is probably a reflection of the increased stability of DHAC compared to the other nucleosides (16, 18).

DHAC and 5-aza-C induced a transient DNA hypomethylation in both cell lines; 7 days after the treatments the methylation levels had returned to the control levels (Fig. 2). However, under the experimental conditions specific changes in methylation at a small number of sites would not have been detected and may have been of a persistent nature. This would permit changes in methylation of the dCk gene in the CEM/dCk⁻ cells to remain undetected.

Although in the CEM/0 cells 5-aza-Cdr and ara-AC were the most potent inducers of hypomethylation, these nucleosides would not be useful in treatment of cells resistant to ara-C due to lack of dCk activity. 5-aza-Cdr and ara-AC showed very low toxicity toward the CEM/dCk⁻ cells (Table 2) with IC_{50} concentrations over 500 times higher than for the CEM/0 cells and even these high concentrations of drugs had no effect on the methylation level.

There are several reports that specific genes have been reactivated after treatment with hypomethylating agents like 5-aza-C (6). We have shown that murine L1210 leukemia cells resistant to ara-C can regain sensitivity to ara-C after treatment with DHAC (28). This was also shown in 2 patients who were clinically resistant to high-dose ara-C and who received 5-aza-C followed by high-dose ara-C (28). Here we show that after exposure to 5-aza-C and DHAC a small proportion of the resistant human leukemia cells reexpress dCk activity. Our results show a direct relationship between induced DNA hypomethylation and reexpression of dCk activity in the CEM/dCk⁻ cell line (Tables 2 and 3). The increase in enzyme activity was up to 0.2 pmol/mg × min, which is 0.2% of the activity in the CEM/0 cells. Reexpression of dCk activity in the CEM/

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