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Differential Nuclear Protein Binding to 5-Azacytosine-Containing DNA as a Potential Mechanism for 5-Aza-2′-Deoxycytidine Resistance

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A clonal cell line (56-42) that was stably and exclusively resistant to the toxic effects of the antileukemic agent 5-aza-2′-deoxycytidine (5-aza-CdR) was derived from C3H 10T1/2 Cl8 cells after multiple treatments with 5-aza-CdR. The 50% lethal dose of 5-aza-CdR for these cells was 1.3 μM, which was 15-fold greater than that for the parental cells. Cell line 56-42 was slightly cross-resistant to the ribo-analog 5-azacytidine, but was sensitive to the nucleoside analog 1-β-D-arabinofuranosylcytosine and to colcemid. Both parental and resistant cell lines incorporated equimolar amounts of 5-aza-CdR into DNA. Resistance was therefore not due to decreased activation, increased detoxification, or reduced incorporation of the drug. The overall level of cytosine methylation in the resistant clone was 80% lower than the level in the sensitive cells. Therefore, the potential number of hemimethylated sites created by the incorporation of equivalent amounts of 5-aza-CdR into the DNA of the two cell types was much greater in the sensitive cells. Furthermore, 5-azacytosine-substituted DNA from the sensitive cells bound 100% more nuclear protein in the form of highly stable complexes. The incorporation of 5-aza-CdR opposite methylated cytosine residues in DNA of the sensitive cells thus resulted in increased nuclear protein binding at hemimethylated sites. This relative increase in tight-binding protein complexes was shown to occur in living cells and may well disrupt replication and transcription and instigate cell death. The differential binding of proteins to hypomethylated, azacytosine-containing DNA may thus mediate a novel mechanism of drug resistance.

The methylation of specific cytosine residues in DNA is thought to play a role in the transcriptional regulation of certain genes in eucaryotic cells (3, 4, 15, 25). The deoxycytidine analog 5-aza-2′-deoxycytidine (5-aza-CdR) has been widely used as an inducer of suppressed genetic information (13, 14, 16). The analog is thought to act by incorporating into DNA (5) where it inhibits the postreplicative methylation of cytosine residues (10, 30). The resulting hypomethylation of the genome has been associated with the activation of certain genes (11, 14).

5-Aza-CdR is also an effective antileukemic agent in both humans (36) and mice (23, 28, 34). The antineoplastic action of the drug is related to its incorporation into DNA (31) and may be mediated through its ability to inhibit DNA methylation (33, 37). In contrast to our present understanding of the mechanisms of 5-aza-CdR-induced gene activation, virtually nothing is known about the mechanisms of drug cytotoxicity. The elucidation of such a mechanism is especially important since the development of resistance to 5-aza-CdR is a major problem in the treatment of leukemias (32).

We previously derived a series of cell lines that were resistant to 5-aza-CdR and reported the initial characterization of these cells (12). In this study, we isolated clones from a mass culture of 5-aza-CdR-resistant cells and characterized the mode of drug resistance so that the mechanism of cytotoxicity can be understood. Resistance to 5-aza-CdR had been attributed previously to decreased activity of deoxycytidine kinase (34, 35), which is the enzyme that phosphorylates 5-aza-CdR before its incorporation into DNA. However, the 5-aza-CdR resistance observed in the isolated clones was not due to decreased drug activation or to increased detoxification of the drug. Instead, we propose that the mechanism of resistance is inherent in the hypomethylating activity of 5-aza-CdR and involves the differential binding of nuclear proteins to hypomethylated, azacytosine-containing DNA.

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MATERIALS AND METHODS

Derivation of cell lines. The 5-aza-CdR-resistant cell lines were derived by repetitive treatment of C3H 10T1/2 Cl8 (10T1/2) cells (26) with increasing concentrations of 5-aza-CdR as previously described (12). Mass cultures were sequentially exposed 10 times to 1 μM 5-aza-CdR, one time to 3 μM 5-aza-CdR, one time to 10 μM 5-aza-CdR, two times to 20 μM 5-aza-CdR, two times to 50 μM 5-aza-CdR, and four times to 100 μM 5-aza-CdR. After these 20 treatments, clones (56-41, 56-42, and 56-43) were isolated from the mass culture by seeding 200 cells per 60-mm-diameter dish. Well-separated, discrete colonies were ring isolated, trypsinized, and grown for further studies.

Growth curves. Cells were seeded into 60-mm-diameter dishes at 20,000 cells per dish and treated 24 h later with 1.0 μM 5-aza-CdR or with 1.0 μM 1-β-D-arabinofuranosylcytosine (ara-C). Control cells were not treated with drug. After 24 h of exposure to the drug, the medium was changed and the cells were allowed to recover. Mean population doublings were calculated by counting the number of cells in duplicate dishes at 24-h intervals after treatment. [Mean population doublings = (ln of number of cells harvested/number of cells seeded)/ln 2.] Each experiment was repeated three times.

Cytotoxicity assays. Cells (200 or 1,000) were seeded into 60-mm-diameter dishes (three to six dishes per treatment) and treated 24 h later with different drug concentrations. After 24 h, the medium was changed and the cells were allowed to grow. Fourteen days after seeding, the cells were fixed with methanol and stained with Giemsa stain, and the number of colonies was counted. Percent survival of treated cells was calculated relative to that of untreated cells.

Incorporation of nucleosides into DNA. The incorporation of nucleosides into DNA was measured as previously described (12). In contrast to naturally occurring bases, 5-azacytosine (5-aza-C) labeled in the 6 position loses its radiolabel after base hydrolysis. 5-Aza-CdR incorporation into DNA was therefore defined as the radioactivity which was insensitive to RNase, precipitable by trichloroacetic acid, but sensitive to alkaline hydrolysis. [*methyl*-^{3}H]thymidine (60 Ci/mmol) was obtained from ICN Pharmaceuticals, Inc. (Irvine, Calif.); [6-^{3}H]-2′-deoxycytidine (1 Ci/mmol) and 5-[6-^{3}H]aza-CdR (9 Ci/mmol) were from Moravek Biochemicals (Brea, Calif.).

Isolation of 0.3 M NaCl nuclear protein extract. The nuclear protein extracts were prepared as described by Christman et al. (8). Logarithmically growing cells (10T1/2 or 56-42) were harvested and washed once in phosphate-buffered saline. Cell pellets were suspended in lysis buffer (0.3% Triton X-100, 0.32 M sucrose, 10 mM Tris hydrochloride [pH 7.8], 3 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) and placed at 4°C for 5 min. The suspension was centrifuged at 2,000 × *g* at 4°C for 10 min, and the supernatant was discarded. The pellet was resuspended in lysis buffer and centrifuged as before, and the resultant nuclear pellet was frozen at −80°C. For nuclear protein extraction, the pellet of nuclei was thawed and suspended in Berkowitz buffer (2) containing phenylmethylsulfonyl fluoride (0.32 M sucrose, 1.0 mM $MgCl_2$, 1.0 mM potassium phosphate [pH 6.8], 0.1 mM phenylmethylsulfonyl fluoride). The suspension was frozen in dry ice-ethanol and then thawed; this process was repeated twice. Sodium chloride (3.0 M) was added to the suspension to a final concentration of 0.3 M, and the suspension was placed at 4°C for 15 min. After centrifugation at 45,000 × *g* at 4°C for 4 h, the resulting supernatant was dialyzed against buffer A (100 mM imidazole [pH 7.5], 20 mM EDTA, 0.5 mM dithiothreitol). The protein content of the nuclear extract was determined by the Bradford assay (6).

For experiments studying the effect of 5-aza-CdR on the extractability of nuclear proteins, cultures of log-phase 10T1/2 or 56-42 cells were treated with 0.3 μM 5-aza-CdR for 24 h before harvesting the cells. The isolation of 0.3 M NaCl nuclear protein extracts from control and 5-aza-CdR-treated cultures was performed as described above.

Preparation of control and 5-aza-C-containing labeled DNA substrates from 10T1/2 and 56-42 cells. Labeled DNA substrates were prepared as described by Christman et al. (8). Logarithmically growing cells (10T1/2 or 56-42) were treated with [^{3}H]thymidine (0.5 μCi/ml) or with [^{3}H]thymidine (0.5 μCi/ml) and 5-aza-CdR (5.0 μM) for 24 h. The cells were then harvested, and DNA was isolated by the method of Marmur (21) with minor modifications (7). The DNA was treated with S1 nuclease (25 units/μg of DNA) to remove any single-stranded DNA that may interfere with subsequent nuclear protein binding reactions. The specific activity of the S1 nuclease-treated DNA was quantitated by trichloroacetic acid precipitation (final concentration, 10%), filtration through nitrocellulose filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.), washing with 5% trichloroacetic acid, and quantitation of the radioactivity by scintillation counting.

Preparation of control and 5-aza-C-containing labeled DNA from *Escherichia coli* B. *E. coli* B was grown to an A_{550} of 0.6 in minimal M9CA media (20) supplemented with vitamin B (1 μg/ml). The control culture was then treated with [^{3}H]thymidine (1 μCi/ml), and the 5-aza-CdR culture was treated with [^{3}H]thymidine (1 μCi/ml) and 5-aza-CdR (10 μg/ml) for one population doubling. During this period, approximately 2% of the total cytosine residues were replaced by 5-aza-C (data not shown). This value of substitution was comparable to the amount in the eucaryotic DNA substrates. The bacteria were harvested by centrifugation at 4,000 × *g* for 10 min. DNA was isolated from the control and the 5-aza-CdR-treated *E. coli* B as described by Marmur (21). The DNA was treated with S1 nuclease (25 units/μg of DNA), and the specific activity of the double-stranded DNA was quantitated as trichloroacetic acid-precipitable radioactivity on 0.45-μm-pore-size nitrocellulose filters.

Filter binding assay. DNA-protein interactions were quantitated by the filter binding assay of Christman et al. (8). Each assay consisted of 5 μg of labeled DNA and 40 to 120 μg of nuclear protein extract in a final volume of 200 μl of buffer A (100 mM imidazole [pH 7.5], 20 mM EDTA, 0.5 mM dithiothreitol). The binding reaction was performed at 37°C for 20 min and was terminated by the addition of Sarkosyl (Sigma Chemical Co., St. Louis, Mo.), and NaCl to final concentrations of 0.6% and 0.5 M, respectively. The reaction mixture was mixed thoroughly, incubated at 4°C for 10 min, and then filtered through a Millipore HA 0.45-μm-pore-size filter. Each filter was washed with three 10-ml volumes of dissociation buffer (0.6% Sarkosyl, 0.5 M NaCl, 10 mM Tris hydrochloride [pH 7.4], 0.1 mM EDTA). The filters were then dried and immersed in Permablend III nonaqueous fluor, and the bound radioactivity was quantitated by liquid scintillation counting.

RESULTS

Derivation of clones 56-41, 56-42, and 56-43. Three clones (56-41, 56-42, and 56-43) were isolated from the mass culture of 10T1/2 cells that had been treated 20 times with increasing concentrations of 5-aza-CdR. Successive drug treatments resulted in progressively decreased levels of overall DNA methylation (12). The methylation levels of the three clones were 0.75, 0.63, and 0.62%, respectively, which were approximately 80% lower than the 3.2% cytosine modification level of the parental untreated 10T1/2 cells (22). The repeated drug treatments allowed us to derive 5-aza-CdR-resistant clones that had drastically decreased levels of cytosine methylation. The methylation status of specific genes in clones 56-41, 56-42, and 56-43 were also markedly decreased, and there were profound changes in the differentiated state of the cells (manuscript in preparation).

Drug resistance of clone 56-42. All three clones were more resistant to the toxic effects of 5-aza-CdR than were the parental 10T1/2 cells, and the level of resistance was similar in all three clones (data not shown). Characterization of the mode of resistance was examined in clone 56-42 as its growth rate was most similar to that of the parental 10T1/2 cells (data not shown).

Colony plating assays showed that clone 56-42 was about 15 times more resistant to 5-aza-CdR than were 10T1/2 cells (Fig. 1). The 50% lethal dose for 56-42 cells was 1.3 μM,

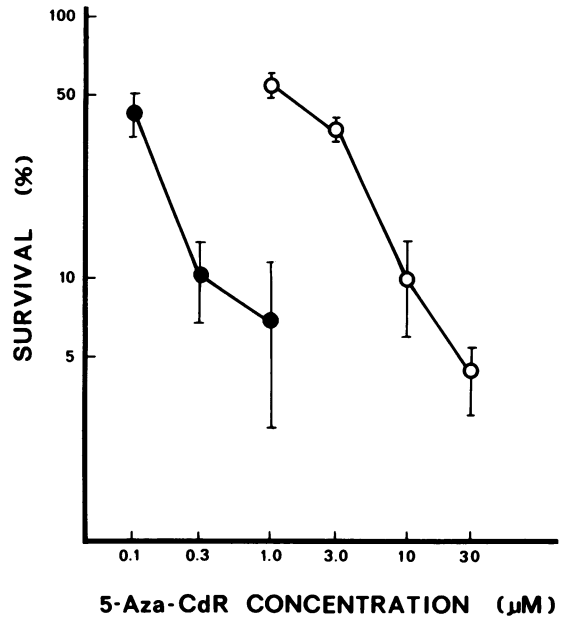

FIG. 1. Toxicity assays for 10T1/2 (●) or 56-42 (○) cells treated with 5-aza-CdR. Cells (200 or 1,000) were seeded into 60-mm dishes and treated 24 h later with the nucleoside for 24 h. Cultures were fixed 14 days after treatment, stained colonies were counted, and the survival of treated cells was compared with that of untreated cells. Each experiment was performed in triplicate, and the results represent the mean ± the standard deviation (bars) of three independent experiments.

whereas that for 10T1/2 cells was 0.09 μM. Furthermore, the resistant phenotype was stable when the cells were passaged at least 35 times in the absence of 5-aza-CdR (data not shown). The cell line 56-42 was, however, only slightly cross-resistant to the ribo-analog 5-azacytidine (5-aza-CR) (Fig. 2). Thus, the induced resistance was specific for 5-aza-CdR.

The nucleoside analog ara-C is metabolized along the same pathway responsible for the incorporation of 5-aza-CdR into DNA. We compared 10T1/2 cells with 56-42 cells for their sensitivities toward ara-C to detect altered activities of the enzymes deoxycytidine kinase and cytidine deaminase (Fig. 3). Both cell lines were equally sensitive to the toxic effects of the analog, suggesting that the resistance of clone 56-42 to 5-aza-CdR was not due to increased detoxification of 5-aza-CdR by cytidine deaminase or to reduced activation of the analog by the kinase enzyme.

The growth inhibitory effects of 5-aza-CdR or ara-C on 10T1/2 and 56-42 cells were also measured (Fig. 4). 5-Aza-CdR had a more pronounced inhibitory effect on the growth rate of 10T1/2 cells than on 56-42 cells. However, ara-C affected the growth rates of both cell lines to similar extents (Fig. 4). These results provided further evidence that the resistance of 56-42 cells to 5-aza-CdR was specific for the aza-deoxynucleoside.

Development of resistance to colcemid has often been associated with an induced multidrug resistance phenomenon (1, 19). We therefore tested the sensitivities of 10T1/2 and 56-42 cells to the alkaloid and found both to be equally sensitive to the drug (Fig. 5). This result suggested that 5-aza-CdR resistance in 56-42 cells was not due to a pleiotropic response.

Incorporation of [^{3}H]5-aza-CdR into cellular DNA was measured to confirm that drug resistance was not due to the failure of 56-42 cells to incorporate the fraudulent base into DNA. Both 10T1/2 cells and 56-42 cells incorporated the same amount of 5-aza-CdR into DNA in a dose-dependent manner (Table 1). These results agreed with our previous data for 5-aza-CdR incorporation into the mass culture of resistant cells (12). At 1 μM 5-aza-CdR, the substitution of cytosine residues by 5-aza-C was 2.05% in both 10T1/2 and 56-42 DNA (Table 1). Clone 56-42 cells could therefore survive (Fig. 1) even with substantial amounts of 5-aza-C in their DNA.

DNA synthesis occurred at the same rate in 10T1/2 and 56-42 cells, as shown by [^{3}H]thymidine incorporation during drug treatment (Table 1). Furthermore, the rate of [^{3}H]de-

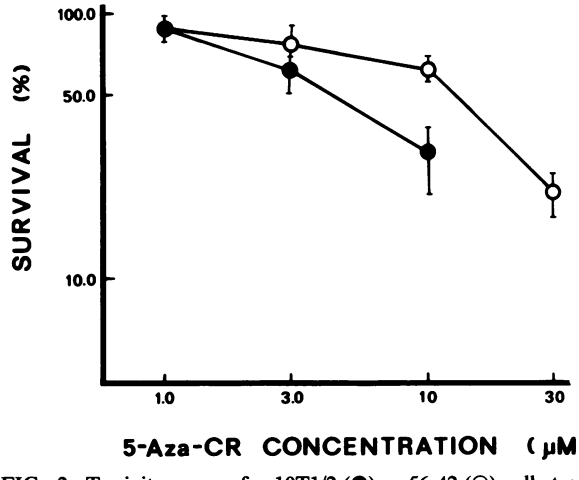

FIG. 2. Toxicity assays for 10T1/2 (●) or 56-42 (○) cells treated with 5-aza-CR. Experimental conditions are as described in the legend to Fig. 1, except that 5-aza-CR was used instead of 5-aza-CdR.

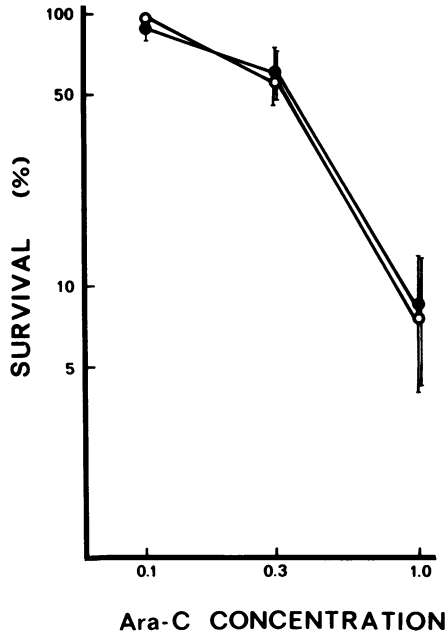

FIG. 3. Toxicity assays for 10T1/2 (●) or 56-42 (○) cells treated with ara-C. Experimental conditions are as described in the legend to Fig. 1.

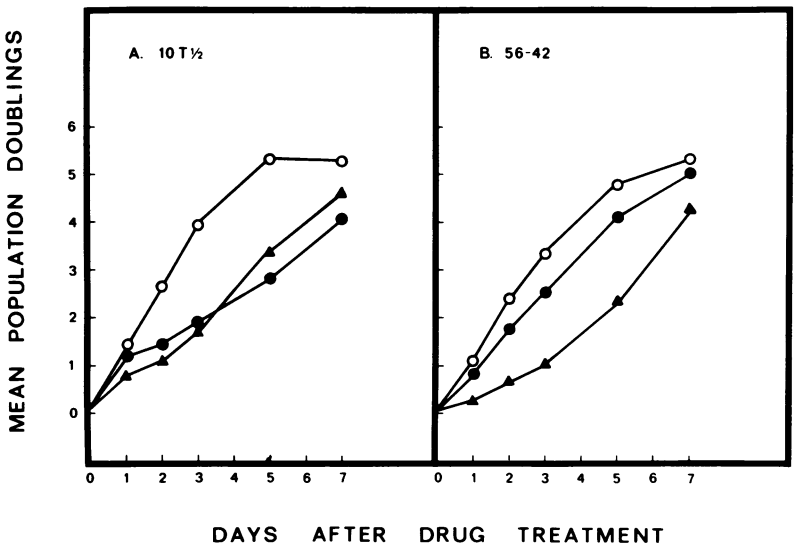

FIG. 4. Effects of 5-aza-CdR or ara-C on the growth of 10T1/2 cells (A) or 56-42 cells (B). Cells (2×10^4) were seeded into 60-mm dishes and treated 24 h later with 1.0 μM 5-aza-CdR (●) or with 1.0 μM ara-C (▲) for a further 24 h. The medium was then changed, and the growth rate was determined by counting the number of cells in duplicate dishes at the indicated times. Data represent one of two experiments which gave the same results. ○, Untreated cells.

oxycytidine incorporation was similar in both cell types (Table 1), substantiating the indirect evidence that the activities of deoxycytidine kinase and cytidine deaminase were similar in both cell lines.

DNA methyltransferase levels. Once incorporated into DNA, 5-aza-CdR causes genome-wide DNA hypomethylation as the analog forms a tight-binding complex with the methyltransferase enzyme (10, 27, 30). We measured the methyltransferase levels in 10T1/2 and 56-42 cells to ascertain whether altered enzyme activities played a role in 5-aza-CdR drug resistance. The average methyltransferase activity of 10T1/2 cells was 6.72 ± 0.28 units and that of 56-42 cells was 7.53 ± 0.88 units (1 unit is defined as 1 pmol of methyl groups transferred from *S*-adenosyl-L-methionine to an artificial DNA substrate per h per mg of DNA per μg of protein) (17). Since both cell lines exhibited similar methyltransferase activities, the resistance could not be attributed to the induction of excessive amounts of methyltransferase that would overcome the 5-aza-CdR-associated enzyme inhibition.

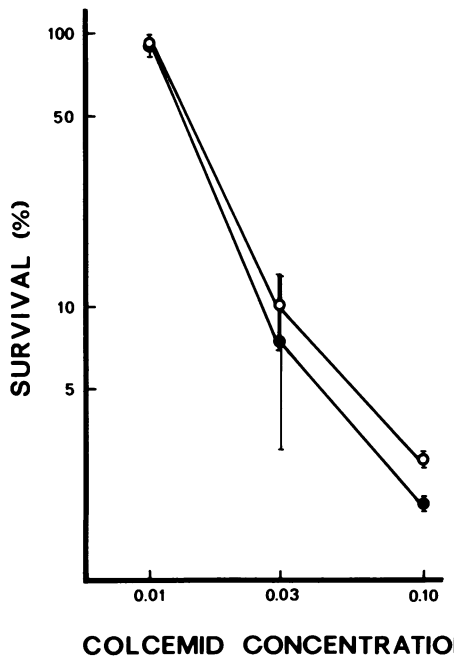

FIG. 5. Toxicity assays for 10T1/2 (●) or 56-42 (○) cells treated with colcemid. Experimental conditions are as described in the legend to Fig. 1.

DNA-protein interactions in resistant cells. Christman et al. (8) elegantly demonstrated that the presence of 5-aza-C in DNA of treated cells resulted in the formation of strongly linked DNA-protein complexes resistant to disruption by Sarkosyl detergent. Such tight-binding complexes may interfere with replication and transcription and lead to cell death. We therefore examined 10T1/2 and 56-42 cells for the levels of nuclear proteins which would bind to 5-aza-C-substituted DNA ([5-aza-C] DNA) to determine whether differential protein binding could play a role in the phenomenon of drug resistance.

The abilities of nuclear proteins extracted from both cell types to form Sarkosyl-resistant complexes with native and

TABLE 1. The incorporation of labeled precursors into DNA of 10T1/2 and 56-42 cells[a]

Precursor	Concn (μM)	Incorporation into DNA (pmol/μg DNA)		% Substitution of cytosine in cells by 5-aza-C	
		10T1/2	56-42	10T1/2	56-42
[^{3}H]5-aza-CdR	0.06	0.94	0.88	0.17	0.15
	1.0	11.72	11.69	2.05	2.05
	10.0	42.96	59.52	7.52	10.43
[^{3}H]thymidine	0.003	0.89	0.75		
[^{3}H]cytidine	1.0	197.96	210.00		

[a] Cells (10T1/2 or 56-42) were seeded at 2×10^5 cells per 60-mm dish and treated 24 h later with the indicated precursor for 24 h. The incorporation of precursors into DNA was determined as previously described (12). Data are given as the mean values for triplicate determinations.

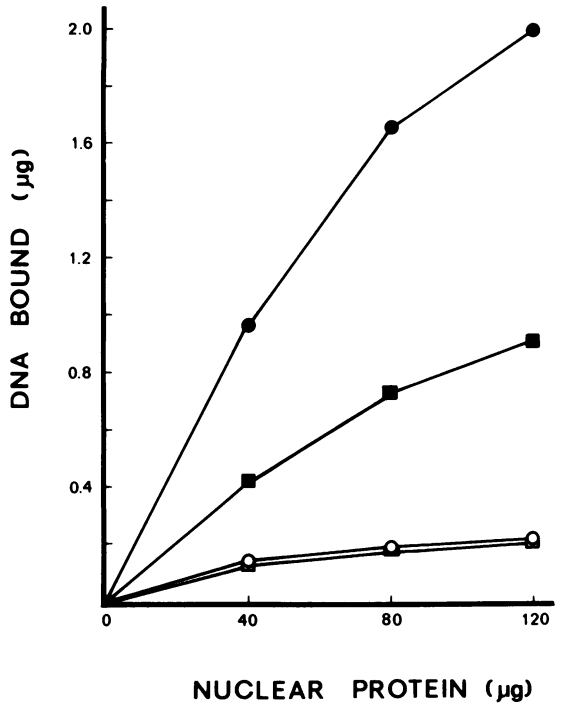

FIG. 6. Effect of protein concentration on the formation of salt- and Sarkosyl-resistant complexes with native 10T1/2 (○), native 56-42 (□), [5-aza-C] 10T1/2 (●), and [5-aza-C] 56-42 (■) DNAs. All reaction mixtures contained 5 μg of [^{3}H]thymidine-labeled, double-stranded DNA and 0 to 120 μg of 0.3 M NaCl protein extract of nuclei from the same cell type. The binding reaction was carried out at 37°C for 20 min and was terminated by the addition of NaCl and Sarkosyl to 0.5 M and 0.6%, respectively, before filtration through nitrocellulose. Complex formation was quantitated by scintillation counting of radioactive DNA retained on the filters. Each value represents the average of duplicate determination.

[5-aza-C] DNA from each cell type was measured by filter binding assays (Fig. 6). The incorporation of 5-aza-CdR into 10T1/2 DNA resulted in a 10-fold increase in the binding of 10T1/2 nuclear proteins to this DNA, as compared with native 10T1/2 DNA. In contrast, there was only a fivefold increase in the binding of 56-42 nuclear proteins to [5-aza-C] 56-42 DNA as compared with native 56-42 DNA. These results confirmed Christman's studies that the incorporation of 5-aza-CdR into DNA resulted in increased nuclear protein binding (8). Furthermore, comparison of the binding levels in the [5-aza-C] DNAs showed that a higher level of tight-binding complexes formed with proteins and DNA from the sensitive 10T1/2 cells than with those from the resistant 56-42 cells. Heterologous filter binding assays were performed next to determine whether the increased binding was due to a higher level of binding proteins or to a difference between the two DNA substrates.

We measured the binding of either 10T1/2 or 56-42 proteins to the same [5-aza-C] DNA substrates (Table 2). The absolute values for the amount of DNA retained on the filters as stable, Sarkosyl-resistant DNA-protein complexes varied with each experiment, but values for triplicate assays within each experiment were highly reproducible (±10%). The ratio between the amount of a given DNA substrate bound by 10T1/2 proteins to that bound by 56-42 proteins was always approximately 1.0 (Table 2). Data from three experiments showed that the average ratio of [5-aza-C] 10T1/2 DNA bound by 10T1/2 nuclear proteins to that bound by 56-42 proteins was 1.19 ± 0.24. Similarly, this ratio was 1.12 ± 0.15 when [5-aza-C] 56-42 DNA was used as the substrate. In other words, both 10T1/2 and 56-42 nuclear protein extracts had the same amounts of protein available for binding to the [5-aza-C] DNA substrates. These results showed that the twofold difference in binding between [5-aza-C] 10T1/2 DNA and [5-aza-C] 56-42 DNA (Fig. 6) was not due to differences in the levels of nuclear proteins available for binding to [5-aza-C] DNA.

On the other hand, the source of [5-aza-C] DNA substantially altered the amount of binding (Table 2). In experiments utilizing 10T1/2 nuclear proteins, the average ratio of 10T1/2 DNA bound versus 56-42 DNA bound was 2.18 ± 0.08 (Table 2). Measuring 56-42 nuclear protein binding to each of the substrates gave a similar average ratio of 2.09 ± 0.18. Therefore, twice as much [5-aza-C] 10T1/2 DNA was bound than [5-aza-C] 56-42 DNA irrespective of the protein extract. Since both cell lines incorporated the same amount of 5-aza-CdR (Table 1) the difference in protein binding could not be attributed to differential incorporation of the fraudulent base.

Significantly, the only detected difference in the DNA sequence between 10T1/2 and 56-42 cells was that the latter had a fivefold lower 5-methylcytosine content (manuscript in preparation). High-pressure liquid chromatographic analysis revealed that the treatment of cells with 5×10^{-6} M 5-aza-CdR and [^{3}H]uridine for one replication cycle resulted in the creation of approximately 1×10^7 hemimethylated sites in 10T1/2 DNA versus 2.1×10^6 hemimethylated sites in 56-42 DNA (data not shown). Therefore, these comparative binding studies suggested that the increased binding was not due to the incorporation of 5-aza-CdR per se, but to the incorporation of the analog opposite a methylcytosine residue.

Filter binding assays with [5-aza-C] *E. coli* B DNA were performed to address the question of whether the increased binding was due to the incorporation of the azanucleoside itself or to the consequential creation of hemimethylated sites containing 5-aza-C. The genome of *E. coli* B does not contain 5-methylcytosine (24), so 5-aza-CdR-induced protein binding could be measured independent of 5-methylcytosine content or hemimethylated sites.

The incorporation of 5-aza-CdR into *E. coli* B DNA did not result in increased formation of salt- and Sarkosyl-resistant DNA-protein complexes (Table 2). Experiments with [^{3}H]5-aza-CdR showed that about 2% of total cytosines were substituted by 5-aza-C (data not shown). These results confirmed our hypothesis that the 5-aza-CdR-induced in-

TABLE 2. Comparative studies of the binding of 10T1/2 or 56-42 nuclear protein extracts to native or [5-aza-C] DNA substrates[a]

Source of DNA substrate	Source of nuclear protein	DNA retained on filter (μg)			
		Expt 1	Expt 2	Expt 3	Expt 4
[5-aza-C] 10T1/2	10T1/2	0.96	1.47	3.09	
[5-aza-C] 10T1/2	56-42	0.68	1.13	3.64	
[5-aza-C] 56-42	10T1/2	0.42	0.70	1.43	
[5-aza-C] 56-42	56-42	0.36	0.55	1.57	
E. coli B	10T1/2				0.18
[5-aza-C] *E. coli* B	10T1/2				0.16

[a] Filter binding assays were performed as described in the legend to Fig. 6, with 80 μg of 0.3 M NaCl nuclear protein extract. Each value represents the average of duplicate determinations in individual experiments.

TABLE 3. Comparative binding assays of 10T1/2 and 56-42 nuclear proteins extracted after treatment of cells with 5-aza-CdR[a]

Source of DNA substrate	Source of nuclear protein	DNA retained on filter (μg)		Extractable binding protein (% control)
		Expt 1	Expt 2	
[5-aza-C] 10T1/2	10T1/2	1.15	1.13	100.0
[5-aza-C] 10T1/2	[5-aza-C] 10T1/2	0.33	0.32	28.5
[5-aza-C] 10T1/2	56-42	1.15	1.12	99.6
[5-aza-C] 10T1/2	[5-aza-C] 56-42	0.65	0.65	56.9

[a] Nuclear proteins were extracted from control cells (10T1/2 or 56-42) or from cells treated with 0.3 μM 5-aza-CdR before 0.3 M NaCl extraction ([5-aza-C] 10T1/2 or [5-aza-C] 56-42). Filter binding assays were performed as described in the legend to Fig. 6, with 40 μg of 0.3 M NaCl nuclear protein extracts. Each value represents the average of duplicate determinations in individual experiments.

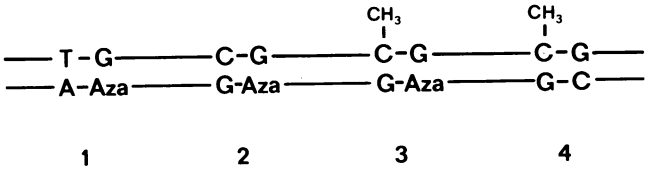

FIG. 7. Schematic representation of the four classes of critical dinucleotide sites created by the incorporation of 5-aza-CdR into DNA during one replication cycle. Class 1, 5-aza-CdR opposite any dinucleotide that is not CpG. Class 2, CpG dinucleotide that is not methylated. Class 3, CpG dinucleotide that is methylated and therefore results in the creation of a hemimethylated site. Class 4, Hemimethylated CpG dinucleotide that does not contain 5-aza-C, but is generated by inhibition of DNA methyltransferase by 5-aza-CdR.

crease in protein binding was due to the inherent ability of the drug to create hemimethylated sites in DNA.

Treatment of living cells with 5-aza-CdR results in the formation of tight-binding complexes which cannot be extracted subsequently with 0.3 M NaCl (10, 30). We therefore determined whether pretreatment of living 10T1/2 or 56-42 cells with the drug before salt extraction would alter the amounts of extractable protein which would subsequently react with [5-aza-C] DNA in the in vitro binding assay (Table 3). Treatment of 10T1/2 cells with 0.3 μM 5-aza-CdR for 24 h decreased the amount of protein capable of forming salt- and Sarkosyl-resistant complexes by 72%. On the other hand, the amount of extractable protein in treated 56-42 cells was only reduced by 43%. These results therefore confirmed the earlier in vitro results and demonstrated a decreased level of DNA-protein complexes in the resistant cells at the drug concentration at which there was an approximately eightfold difference in drug sensitivity (Fig. 1).

DISCUSSION

We derived three cell lines which were markedly resistant to the toxic effects of 5-aza-CdR. Our studies showed that the drug resistance was specific for the analog 5-aza-CdR and was not due to decreased incorporation of 5-aza-CdR into the DNA of resistant cells. Toxicity assays and growth rate studies with ara-C demonstrated that the resistance could not be attributed to decreased levels of deoxycytidine kinase, which is the enzyme responsible for primary activation of the analog, or to increased deaminase activity which could detoxify the fraudulent base. Moreover, resistance was not a pleiotropic response since cells resistant to 5-aza-CdR were highly sensitive to colcemid.

Resistance was not due to altered methyltransferase levels or to decreased levels of nuclear proteins capable of forming tight-binding interactions with [5-aza-C] DNA. Rather, drug resistance appeared to be inherent in the hypomethylating action of 5-aza-CdR and the consequential altered interactions between nuclear proteins and [5-aza-C] DNA.

In both cell lines studied, there are four classes of critical sites created by the incorporation of 5-aza-CdR into DNA during one replication cycle and which would be present in the [5-aza-C] DNA used in our binding assays (Fig. 7). The majority of sites into which the analog is incorporated are not CpG dinucleotides and therefore are not relevant to DNA methylation (class 1). Alternatively, the analog could be incorporated opposite a CpG doublet that is not methylated (class 2). The third potential site of incorporation could be opposite a CpG doublet in which the cytosine is methylated and to which the methyltransferase enzyme could be strongly bound. The formation of such a complex could well inhibit the postreplicative methylation of cytosine residues downstream from the site of 5-aza-CdR incorporation so that the DNA substrates that we used also contained hemimethylated (class 4) sites that did not contain 5-aza-C (27).

Significantly, the 5-methylcytosine content of 56-42 cells was 80% lower than that of 10T1/2 cells. The target size for the creation of hemimethylated sites in 56-42 cells was therefore greatly reduced (Fig. 8). In other words, the treatment of 56-42 cells with 5-aza-CdR would result in an excess of unmethylated class 2 sites relative to 10T1/2 cells. Conversely, 5-aza-CdR treatment of 10T1/2 cells would induce a comparative excess of hemimethylated class 3 or class 4 sites.

We propose that this relative excess of hemimethylated class 3 and class 4 sites in 10T1/2 cells results in increased nuclear protein binding to these sites. The relative increase in nuclear protein binding to [5-aza-C] 10T1/2 DNA was confirmed in living cells treated with 5-aza-CdR. The formation of such tight-binding DNA-protein complexes may interfere with replication in 10T1/2 cells and contribute to cell death. In contrast, the incorporation of the same amount of 5-aza-CdR into 56-42 cells would result in fewer hemimethylated sites and consequently less protein binding and less disruption of macromolecular function so that the cells would be resistant to the drug.

The cellular 5-methylcytosine content and the creation of hemimethylated sites appear to be crucial to the 5-aza-CdR-induced increase in nuclear protein binding, as suggested by binding studies with *E. coli* B DNA substrates (Table 2). Using DNA from a different bacterial source, Christman et al. (9) showed that incorporation of 5-aza-CR into the bacterial DNA did result in increased formation of NaCl- and

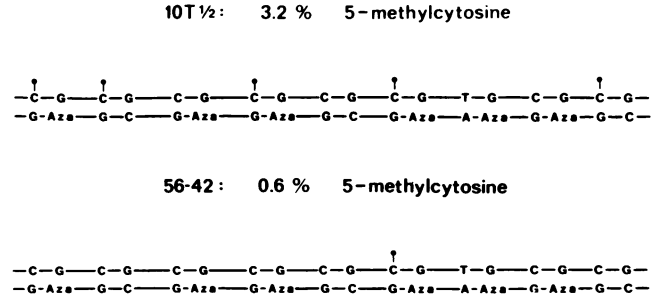

FIG. 8. Schematic representation of the number of hemimethylated sites resulting from the incorporation of equimolar amounts of 5-aza-CdR into 10T1/2 and 56-42 DNA during one replication cycle.

Sarkosyl-resistant complexes with Friend erythroleukemia nuclear protein extracts. The discrepancy between our results and those of Christman et al. (9) may be due to experimental differences such as the percent substitution of 5-aza-C in the bacterial genome.

Our hypothesis that 5-aza-CdR toxicity is at least partly due to the formation of strong DNA-protein interactions is particularly interesting in view of the work of Sugiyama et al. (29). These investigators used the alkaline elution technique (18) to demonstrate that DNA-protein cross-links contributed to the cytotoxic properties of calcium chromate in mammalian cells.

Our results suggest that cellular sensitivity to 5-aza-CdR is mediated in part by alterations in the interactions between DNA and nuclear proteins. The proteins involved in such interactions may include regulatory factors as well as enzymes associated with DNA. Interestingly, the methyltransferase enzyme was identified as one of the proteins in these complexes (8). Further characterization of the biochemical nature of these DNA-protein interactions may elucidate the exact mechanism of 5-aza-CdR-induced cell lethality.

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LITERATURE CITED

1. **Akiyama, S. I., A. Fojo, J. A. Hanover, I. Pastan, and M. M. Gottesman.** 1985. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. Somatic Cell Mol. Genet. **11**:117–126.
2. **Berkowitz, D. M., T. Kakefuda, and M. B. Sporn.** 1969. A simple and rapid method for the isolation of enzymatically active HeLa cell nuclei. J. Cell Biol. **42**:851–855.
3. **Bird, A. P.** 1984. DNA methylation—how important in gene control? Nature (London) **308**:503–504.
4. **Bird, A. P.** 1986. CpG-rich islands and the function of DNA methylation. Nature (London) **321**:209–213.
5. **Bouchard, J., and R. L. Momparler.** 1983. Incorporation of 5-aza-2′-deoxycytidine-5′-triphosphate into DNA: interactions with mammalian DNA polymerase 1 and DNA methylase. Mol. Pharmacol. **24**:109–114.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Chandler, L. A., Y. A. DeClerck, E. Bogenmann, and P. A. Jones.** 1986. Patterns of DNA methylation and gene expression in human tumor lines. Cancer Res. **46**:2944–2949.
8. **Christman, J. K., N. Schneiderman, and G. Acs.** 1985. Formation of highly stable complexes between 5-azacytosine-substituted DNA and specific non-histone nuclear proteins. Implications for 5-azacytidine-mediated effects on DNA methylation and gene expression. J. Biol. Chem. **260**:4059–4068.
9. **Christman, J. K., N. Schneiderman, and G. Acs.** 1985. Interaction of DNA methyltransferase and other non-histone proteins isolated from Friend erythroleukemia cell nuclei with 5-azacytosine residues in DNA, p. 105–118. *In* G. L. Cantoni and A. Razin (ed.), Biochemistry and biology of DNA methylation. Alan R. Liss, Inc., New York.
10. **Creusot, P., G. Acs, and J. K. Christman.** 1982. Inhibition of DNA methyltransferase and induction of Friend erythroleukemic cell differentiation by 5-azacytidine and 5-aza-2′-deoxycytidine. J. Biol. Chem. **257**:2041–2048.
11. **Doerfler, W.** 1983. DNA methylation and gene activity. Annu. Rev. Biochem. **52**:93–124.
12. **Flatau, E., F. A. Gonzales, L. A. Michalowsky, and P. A. Jones.** 1984. DNA methylation in 5-aza-2′-deoxycytidine-resistant variants of C3H/10T1/2 cells. Mol. Cell. Biol. **4**:2098–2102.
13. **Jones, P. A.** 1984. Gene activation by 5-azacytidine, p. 165–187. *In* A. Razin, H. Cedar, and A. D. Riggs (ed.), DNA methylation. Biochemistry and biological significance. Springer-Verlag, New York.
14. **Jones, P. A.** 1985. Altering gene expression with 5-azacytidine. Cell **40**:485–486.
15. **Jones, P. A.** 1986. DNA methylation and cancer. Cancer Res. **46**:461–466.
16. **Jones, P. A., and S. M. Taylor.** 1980. Cellular differentiation, cytidine analogs and DNA methylation. Cell **20**:85–93.
17. **Jones, P. A., and S. M. Taylor.** 1981. Hemimethylated duplex DNAs prepared from 5-azacytidine treated cells. Nucleic Acids Res. **9**:2933–2947.
18. **Kohn, K. W., R. A. G. Ewig, L. C. Erickson, and L. A. Zwelling.** 1981. Measurement of strand breaks and cross-links by alkaline elution, p. 379–401. *In* E. Friedberg and P. C. Hanawalt (ed.), DNA repair: a laboratory manual of research procedures, vol. 1, part B. Marcel Dekker, New York.
19. **Ling, V.** 1985. Multidrug-resistant mutants, p. 773–787. *In* M. M. Gottesman (ed.), Molecular cell genetics. John Wiley & Sons, Inc., New York.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acids from micro-organisms. J. Mol. Biol. **3**:208–218.
22. **Michalowsky, L. A., and P. A. Jones.** 1986. DNA methylation and drug resistance in variants of C3H/10T1/2 Cl 8 cells, p. 391–399. *In* G. Serrero and J. Hayashi (ed.), Cellular endocrinology: hormonal control of embryonic and cellular differentiation. Alan R. Liss, Inc., New York.
23. **Momparler, R. L., and F. A. Gonzales.** 1978. Effect of intravenous infusion of 5-aza-2′-deoxycytidine on survival time of mice with L1210 leukemia. Cancer Res. **38**:2673–2678.
24. **Nikolskaya, I. I., Z. G. Tkatcheva, B. F. Vanyushin, and T. I. Tikchonenko.** 1968. On the presence of minor bases in DNA of phages DD7 and Sd and their hosts. Biochim. Biophys. Acta **155**:626–629.
25. **Razin, A., and M. Szyf.** 1984. DNA methylation patterns. Formation and function. Biochim. Biophys. Acta **782**:331–342.
26. **Reznikoff, C. A., D. W. Brankow, and C. Heidelberger.** 1973. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res. **33**:3231–3238.
27. **Santi, D. V., C. E. Garrett, and P. J. Barr.** 1983. On the mechanism of inhibition of DNA-cytosine methyltransferases by cytosine analogs. Cell **33**:9–10.
28. **Sorm, F., and J. Vesely.** 1968. Effect of 5-aza-2′-deoxycytidine against leukemic and hemopoietic tissues in AKR mice. Neoplasma **15**:339–343.
29. **Sugiyama, M., S. R. Patierno, O. Cantoni, and M. Costa.** 1986. Characterization of DNA lesions induced by $CaCrO_4$ in synchronous and asynchronous cultured mammalian cells. Mol. Pharmacol. **29**:606–613.
30. **Taylor, S. M., and P. A. Jones.** 1982. Mechanism of action of eukaryotic DNA methyltransferase. Use of azacytosine-containing DNA. J. Mol. Biol. **162**:679–692.
31. **Vesely, J., and A. Cihak.** 1977. Incorporation of a potent antileukemic agent, 5-aza-2′-deoxycytidine, into DNA of cells from leukemic mice. Cancer Res. **37**:3684–3689.
32. **Vesely, J., and A. Cihak.** 1978. 5-Azacytidine: mechanism of action and biochemical effects in mammalian cells. Pharm. Ther. **2**:813–840.
33. **Vesely, J., A. Cihak, and F. Sorm.** 1967. Biochemical mechanisms of drug resistance, development of resistance to 5-azacytidine and simultaneous depression of pyrimidine metabolism in leukemic mice. Int. J. Cancer **2**:639–646.
34. **Vesely, J., A. Cihak, and F. Sorm.** 1968. Characteristics of mouse leukemic cells resistant to 5-azacytidine and 5-aza-2′-deoxycytidine. Cancer Res. **28**:1995–2000.

35. **Vesely, J., A. Cihak, and F. Sorm.** 1970. Association of decreased uridine and deoxycytidine kinase with enhanced RNA and DNA polymerase in mouse leukemic cells resistant to 5-azacytidine and 5-aza-2'-deoxycytidine. Cancer Res. **30:** 2180–2186.
36. **Von Hoff, D. D., M. Slavik, and F. M. Muggia.** 1976. 5-Azacytidine, a new anticancer drug with effectiveness in acute myelogenous leukemia. Ann. Intern. Med. **85:**237–245.
37. **Wilson, V. L., R. L. Momparler, and P. A. Jones.** 1983. Inhibition of DNA methylation of L1210 leukemic cells by 5-aza-2'-deoxycytidine: a possible mechanism of chemotherapeutic action. Cancer Res. **43:**3493–3496.