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Long Term Instability and Molecular Mechanism of 5-Azacytidine-Induced DNA Hypomethylation in Normal and Neoplastic Tissues *in Vivo*

LEE-JANE WANG LU AND KURT RANDERATH

Department of Pharmacology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030

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SUMMARY

We have previously shown that treatment of normal and neoplastic cells with the antileukemic drug, 5-azacytidine, led to the rapid synthesis of a low molecular weight RNA containing 5-azacytosine. This fraudulent RNA inhibited tRNA (cytosine-5)-methyltransferase early after drug administration. The absence of tRNA (cytosine-5)-methyltransferase activity resulted in the synthesis of tRNA specifically deficient in 5-methylcytosine. Here, we show that treatment of L1210 cells, grown intraperitoneally in mice, with 5-azacytidine led to a rapid and prolonged inactivation of DNA (cytosine-5)-methyltransferase activity and to the synthesis of undermethylated DNA. DNA isolated from the treated tissue was found to inactivate the DNA methylase (decreased V_{max}) in *in vitro* DNA (cytosine-5)-methyltransferase assays. Kinetic analysis showed noncompetitive inhibition of the substrate by the inhibitor. The persistence of DNA undermethylation after treatment with 5-azadeoxycytidine or 5-azacytidine in animals has not been measured directly; therefore, we have investigated this phenomenon in the intact animal. Prolonged treatment with 5-azacytidine was required to maintain a fraction of undermethylated sites in DNA of L1210 cells *in vivo* for up to 4 months or longer after drug withdrawal. Such treatment led to instability of DNA methylation levels in L1210 cells *in vivo*. At least a partial restoration of DNA 5-methylcytosine levels was observed after acute and chronic 5-azacytidine treatment, respectively. 5-Azacytidine was also found to induce DNA hypomethylation in regenerating, but not in normal adult mouse liver cells. Our results show that: 1) it was extremely difficult to decrease the DNA methylation level to <50% of control; and 2) it was also difficult to maintain stable DNA methylation levels *in vivo* after exposure to the drug.

INTRODUCTION

The antimetabolite, 5-aza-CR,[1] a cytidine analogue in which ring carbon 5 is replaced with nitrogen, is effective in the treatment of acute myelogenous leukemia (1, 2). The drug exhibits a multitude of biological effects in various systems. These include mutagenesis, induction of chromosome breakage, interference with processing of ribosomal RNA, and inhibition of synthesis of DNA, RNA, and protein (3). The analogue is also incorporated into nucleic acids (4, 5). However, the most specific effect of the drug is its interference with the postsynthetic modification of nucleic acids (6–10). In 1976, we demonstrated that 5-aza-CR and another 5-substituted cytidine analogue, 5-fluorocytidine, specifically inhibited the synthesis of m^5C in tRNA (6, 11). Since the amount of analogue incorporation could not account for the extent of inhibition of m^5C synthesis in tRNA, we postulated that the drug inhibited the transfer of methyl groups from SAM to specific cytidine residues in tRNA (6). This mode of action was subsequently proven (7), and an inhibitor of the tRNA m^5C methylase, i.e., a low molecular-weight RNA (probably pre-tRNA containing the incorporated analogue), was identified (8). Since analogous biosynthetic pathways have been proposed for the synthesis of m^5C in both tRNA and DNA of eukaryotic cells, i.e., a postsynthetic modification of the respective macromolecule by a specific methylase which transfers the methyl group from SAM to the 5-position of cytosine (12), we hypothesized that the analogue might also inhibit the synthesis of m^5C in DNA in analogous fashion (6). Subsequently, the drug treatment was reported to decrease DNA methylation in Ehrlich ascites cells (13), cultured 10T1/2 cells (10), and Friend erythroleukemia

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[1] The abbreviations used are: 5-aza-CR, 5-azacytidine; 5-aza-C, 5-azacytosine; m^5C, 5-methylcytosine; C, cytosine; A, adenine; G, guanine; dpNp, deoxyribonucleoside 3′,5′-bisphosphate; dpN, deoxyribonucleoside 5′-phosphate; dNp, deoxyribonucleoside 3′-phosphate; DNA m^5C methylase, DNA (cytosine-5)-methyltransferase; tRNA m^5C methylase, tRNA (cytosine-5)-methyltransferase; SAM, *S*-adenosyl-L-methionine; PEI, polyethylenimine.

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cells (14). Drug treatment was shown to decrease DNA m^5C methylase (EC 2.1.1.37) activity (13, 14). DNA containing 5-aza-C isolated from drug-treated *Escherichia coli* was found to inactivate *E. coli* DNA m^5C methylase *in vitro* (15). In the present paper, we describe the isolation of a DNA fraction which inhibits DNA m^5C methylase *in vitro* from L1210 cells treated with 5-aza-CR *in vivo* and report kinetic data illustrating the mechanism of inhibition of DNA m^5C methylase by the inhibitor.

Several lines of evidence suggest the importance of DNA methylation for the control of gene expression in eukaryotic cells (16). First, gene activity correlates with hypomethylation of control regions (17–19), and *in vitro* methylation of DNA reduces *in vivo* gene activity (20, 21). Second, agents such as ethionine (22) and 5-aza-CR (10, 13, 15), which inhibit DNA methylation in cultured mammalian cells, are able to induce heritable alterations of gene expression in mammalian cells, and the capacity of these analogues to elicit differentiation correlates with their ability to cause undermethylation in DNA (16, 22–25). Third, aberrant DNA methylation levels were found to be associated with numerous neoplastic tissues (16, 26, 27), linking abnormal phenotypic expression with altered DNA methylation. Thus, it appeared important to investigate if an effective antineoplastic and carcinogenic compound, such as 5-aza-CR, would also induce persistent DNA hypomethylation in normal and neoplastic tissues of experimental animals. This question appeared relevant for our understanding of the therapeutic and toxic effects of inhibitors of DNA methylation in humans. In the present study, the effects of 5-aza-CR on DNA methylation in normal and regenerating mouse liver, and L1210 cells grown intraperitoneally were investigated. We observed that it was extremely difficult to decrease the DNA methylation level in liver and in L1210 cells to <83 and <50%, respectively, of control, that only a fraction of the unmethylated sites could be maintained indefinitely *in vivo*, and that prolonged exposure to the analogue resulted in a chronic instability of DNA methylation levels of L1210 cells.

MATERIALS AND METHODS

Materials

RNase A, α-amylase, and 5-aza-CR were from Sigma Chemical Co., St. Louis, MO. Spleen phosphodiesterase was from Worthington Biochemical Co., Freehold, NJ. Nuclease P1 was from Yamasa Shoyu Co., Tokyo, Japan. Proteinase K was from Boehringer Mannheim, Indianapolis, IN. Nonidet P-40 was from Bethesda Research Laboratories, Inc., Gaithersburg, MD. [CH_3-^{3}H]SAM (10–15 Ci/mmol) was from New England Nuclear, Boston, MA. [γ-^{32}P]ATP was from Amersham, Arlington Heights, IL. Deoxyribonucleotides and T4 polynucleotide kinase were from P-L Biochemicals, Milwaukee, WI. Glass fiber filter discs (GF/A) and Partisil 10-SCX columns were from Whatman Chemical Co., Clifton, NJ. PEI-cellulose (Polygram Cel PEI) thin layers were from Brinkmann Instruments, Westbury, NY. A 344 liquid chromatograph with a No. 160 absorbance detector was from Beckman, Berkeley, CA. A prepacked guard column 10-SCX (5 μm, 4.6 × 20 mm) was from Custom LC, Inc., Houston, TX. A reporting integrator No. 3390A was from Hewlett-Packard Co., Avondale, PA. DBA-2 mice were purchased from Charles River Farms, Wilmington, MA, and BDF_1 mice were from Simonson Laboratories, Gilroy, CA. BALB/c Crg1 mice were bred and maintained at the mouse colony of the Department of Cell Biology, Baylor College of Medicine, Houston, TX.

Animal Treatments

Single and short term treatments. Groups of male BDF_1 mice (6–8 mice per group) received intraperitoneal transplants of 1.2×10^5 L1210 cells and 6 days later were given 10.0 mg/kg of 5-aza-CR intraperitoneally, freshly dissolved in 0.9% NaCl. Control mice received 0.9% NaCl treatment. Cells were harvested from the peritoneal cavity at various times after drug treatment.

For long term observation studies, male DBA-2 mice (20 g) received intraperitoneal transplants of 1×10^6 cells and were then treated with 5-aza-CR (1.0 mg/kg) intraperitoneally once a day for 4 days beginning 3 days after transplantation. Cells were harvested 2 days after the last drug treatment. Thereafter, 1×10^5 cells were transplanted to new hosts once a week for another 17 weeks without further drug treatment (Fig. 4).

Long term exposure to the drug. L1210 cells grown intraperitoneally in male DBA-2 mice were exposed to 5-aza-CR administered intraperitoneally continuously during serial transplantations. Table 1 shows the detailed treatment schedules. Each transplant cycle began with the transplantation of 1×10^6 cells (3–6 mice per group) and ended with the harvesting of the cells. Each treatment began 3 days after transplantation. Cells were harvested 2 days after the last drug treatment and retransplanted to new hosts on the same day. The treatment cycle (Table 1) refers to 2–6 doses of 5-aza-CR given to the tumor-bearing mice during one transplant cycle. Because of severe cytotoxicity to the cells, prolonged and continuous exposure of the cells to 5-aza-CR was avoided and doses of the drug were adjusted occasionally to keep the cells viable for long term observations. Thus, the cells were exposed to 5-aza-CR for up to 14 treatment cycles during the 18 transplant cycles shown in Table 1. Cells were withdrawn from further drug treatment after exposure to 5-aza-CR as specified. After drug withdrawal, 1×10^5 cells were given to each mouse and retransplantations were performed once a week for up to 30 weeks (Fig. 5A–D).

Treatments for studies on DNA methylation in mouse livers. Male BALB/c Crg1 mice (20–25 g) were partially hepatectomized (60% of the liver removed) between 9:00 and 11:00 a.m. under light ether anesthesia. Various doses of 5-aza-CR were given 24 hr after partial hepatectomy. Mice were killed by cervical dislocation. Livers were

TABLE 1

Detailed schedule for long-term treatment of L1210 cells, grown intraperitoneally, with 5-aza-CR

Male DBA-2 mice received transplants of 1×10^6 cells and were treated with the drug starting 3 days after transplantation. Cells were harvested 2 days after the last treatment and retransplanted to new hosts on the same day for continuing treatment (see text).

Transplant number	Doses	Treatment on days after transplantation[a]
	mg/kg	
1–4	1.0	3, 4, 5, 6
5–7[b]		
8	1.0	3, 4, 5
9 & 10	1.0	3, 4, 5, 6
11	1.0	3, 5
12 & 13	0.5	3, 4, 5, 6, 7, 8
14[b]		
15	0.5	3, 4, 5, 6
16	1.0	3, 4, 5, 6
17	1.0	3, 6
18	1.0	3, 5, 7, 9

[a] The drug was withdrawn after the 4th (Fig. 5A), 12th (Fig. 5B), 13th (Fig. 5C), and 18th (Fig. 5D) transplant, respectively.

[b] No drug was given during 5th, 6th, 7th and 14th transplants.

removed at various times after the last drug treatment. Tissues were kept at −80° until DNA isolation.

Isolation of Nuclei

L1210 cells harvested from the peritoneal cavity of DBA-2 or BDF_1 mice were pelleted at 2700 rpm for 5 min and resuspended in 20 volumes of 0.9% NaCl and pelleted again. Cells were then homogenized in 40 volumes of reticulocyte lysate buffer (10 mM Tris-HCl, 10 mM NaCl, 10 mM $Mg(OAc)_2$, pH 7.4) containing 0.5% Nonidet P-40. The homogenate was underlayered with an equal volume of 0.8 M sucrose and centrifuged at 2700 rpm for 20 min. The pelleted nuclei were frozen at −80° until use for isolation of DNA methylase or DNA.

DNA Isolation from L1210 Cells or Nuclei

Approximately 0.5 ml of packed whole cells or nuclei was suspended in 40 ml of saline-versene buffer (0.015 M NaCl, 0.01 M EDTA, pH 8.0) containing 0.1 mg/ml proteinase K and 1% sodium dodecyl sulfate. Incubation was at 38° for 2 hr with shaking. After two deproteinizations with chloroform/isoamyl alcohol/phenol (90:5:5, by volume), DNA was recovered from the solution by spooling in the presence of 2 volumes of ethanol containing 2% potassium acetate (pH 5.1). The DNA was dissolved in saline-versene and further treated with 0.1 mg/ml each of RNase A and α-amylase at 38° for 2 hr, and finally incubated with 50 μg/ml of proteinase K at 38° for 2 hr. After deproteinization and spooling as described above, DNA was used for base composition analysis to estimate the extent of hypomethylation. DNAs used for base analysis were in some instances further hydrolyzed in 0.5 M NaOH at 60° for 0.5 hr to ensure removal of residual RNA. However, this treatment was found to have no effect on the DNA base compositions.

DNA Isolation from Liver

Frozen livers were pulverized and homogenized in 10 volumes of 0.3 M sucrose containing 10 mM EDTA, pH 8.0, with 10 up-and-down strokes in a Potter-Elvehjem homogenizer. The homogenate was then incubated in a final volume of 40× the tissue weight with 0.1 mg/ml proteinase K, 1% sodium dodecyl sulfate, and 10 mM EDTA, pH 8.0, at 38° for 2 hr. Deproteinization, RNase A, and α-amylase treatments described above were then performed to purify the DNA.

Analysis of DNA Nucleotide Composition by ^{32}P-Labeling

The nucleotide composition of minute amounts of DNA (1–2 μg) was analyzed by the procedure of Reddy *et al.* (28), except that the solvent used for development in the first dimension was 0.5 M ammonium formate, pH 3.5, developed for 18 hr onto a Whatman No. 1 wick. The nucleotide composition was calculated by dividing the cpm in each nucleotide by the total cpm in all nucleotides and expressed as a percentage of the total.

Determination of m^5C Content by HPLC

Whenever sufficient DNA was available, DNA m^5C content was determined by HPLC as described (27). DNA was hydrolyzed in concentrated hydrofluoric acid (300 μl hydrofluoric acid/50 μg of DNA) for a minimum of 4 hr according to the procedure of Lapeyre *et al.* (26). Bases were then analyzed by HPLC on a Beckman 344 liquid chromatograph with a Beckman 160 absorbance detector. Base separation was carried out on a cation-exchange Partisil 10-SCX column coupled with a prepacked SCX guard column. Bases were eluted isocratically with 0.25 M ammonium formate, pH 3.2, at a flow rate of 1.0 ml/min. Their amounts were quantitated electronically using Hewlett-Packard Reporting Integrator 3390A. Each DNA sample was subjected to at least two acid hydrolyses, and at least two analyses were performed for each acid hydrolysate. m^5C content of DNA was expressed as mole % of m^5C/(mole % of m^5C + mole % of C), unless otherwise specified. The m^5C content of control L1210 cell DNA was 0.0352 ± 0.009 (five analyses) and 0.0376 ± 0.0006 (four analyses) by ^{32}P-labeling and by HPLC analyses, respectively.

DNA m^5C Methylase Preparation

The enzyme was isolated according to the procedure of Tanaka *et al.* (13) with some modifications. Briefly, frozen nuclei were suspended in 5 volumes of DNA methylase buffer (50 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, pH 7.8). The $[Na^+]$ concentration was then adjusted to 0.4 M with 4 M NaCl. The mixture was stirred for 20 min, centrifuged at 105,000 × *g* for 30 min, and then the supernatant solution was removed. Prewashed (29) DEAE-cellulose was added to the solution to give a 25% (w/v) slurry, and DEAE-cellulose was removed by filtration. The filtrate was adjusted to 30% saturation with ammonium sulfate. The precipitate was discarded and the supernatant solution was adjusted to 60% saturation with ammonium sulfate. The resulting precipitate was taken up in a small volume of DNA methylase buffer and dialyzed exhaustively against this buffer. Protein concentration was determined according to Lowry's procedure (30).

DNA m^5C Methylase Assays

Substrate DNA used for this assay was isolated from L1210 cells treated *in vivo* with 4.0 mg/kg of 5-aza-CR for 24 hr. For determining enzyme activity, the reaction mixture consisted of 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 5 μM $[CH_3$-$^3H]$SAM (10–15 Ci/mmol), 50 μg of enzyme protein, 25 μg of substrate DNA, and 1 μg of RNase A (freed of DNase activity by heating at 80° for 10 min) in a final volume of 200 μl. Incubation was at 37° for 1 hr; then 50 μg of pronase was added and incubation was continued for 30 min. The reaction mixture was made 0.5 N with respect to NaOH and incubated at 60° for 30 min. The *methyl*-^3H-labeled DNA was precipitated from 5% trichloroacetic acid and collected by filtration onto a glass fiber filter disc (Whatman GF/A). After thorough washing, the filter was dried and counted in a Packard liquid scintillation counter using a toluene scintillator (0.5% 2,5-diphenyloxazole in toluene). To identify the product as m^5C, the filter disc was removed from the counting solution, rinsed with toluene, and air dried. The filter was then soaked in 2 ml of 2 N NH_4OH to extract DNA. After neutralization of the resulting solution with 1 N HCl, DNA was precipitated from 50% alcohol, 1% KOAc, pH 5.1. The precipitate was dissolved in 5 μl of H_2O and digested with nuclease P1 (1 unit) in 30 μM $ZnCl_2$, 14 mM NaOAc, pH 5.0, in a final volume of 100 μl. After incubation at 38° for 1 hr, the digest was spotted on a PEI-cellulose thin layer that had been prewashed in methano-ammonia (1000:1, v/v) for 5 min. Approximately 50–100 μg of DNA digest in 100 μl could be spotted in 5-μl portions without intermediate drying. After drying, the chromatogram was first developed in methano-H_2O (1:4, v/v) to the origin (2.5 cm from the bottom of the sheet), then in 0.1 N acetic acid to 19 cm above the origin, and finally in 1.0 N HOAc to 5 cm on a Whatman No. 1 wick. The $[^3H]$dpm^5C spot was visualized by fluorography and its identity was established by co-chromatography with reference compound.

Studies on Inhibitors of DNA m^5C Methylase Activity

DNAs isolated from control L1210 cells and from L1210 cells treated with varying doses of 5-aza-CR for 4 hr were screened for possible inhibitory effects on DNA m^5C methylation in the *in vitro* assay described above. A solution of the DNA to be tested (1–25 μg) was either added to the enzyme assay mixture together with the substrate DNA or preincubated at 38° with enzyme and $[CH_3$-$^3H]$SAM for 7 min unless otherwise specified, prior to the addition of substrate DNA. Incubation was at 38° for 60 min after the addition of the latter.

RESULTS

Effects of Single 5-aza-CR Treatments on L1210 Cell DNA m^5C Content and Methylase Activity

Figure 1 shows that a single treatment of L1210 cells grown intraperitoneally with 10.0 mg/kg of 5-aza-CR decreased DNA m^5C content in a time-dependent man-

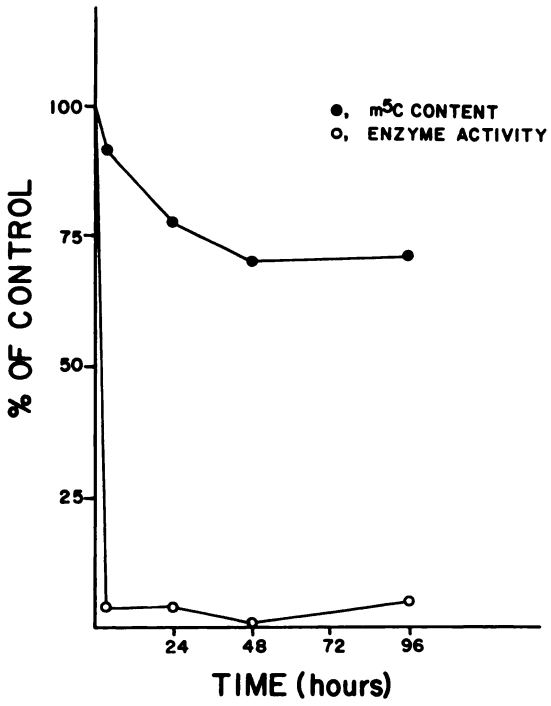

FIG. 1. *Effects of acute treatment of L1210 cells with 5-aza-CR on DNA m⁵C content and DNA m⁵C methylase activity of L1210 cells as a function of time*

DNAs and methylases were isolated from nuclear pellets of L1210 cells grown intraperitoneally in male BDF_1 mice after treatment with 10.0 mg/kg of 5-aza-CR for the indicated lengths of time. Base compositions of L1210 cell DNA were determined by ^{32}P-postlabeling assay. At least five chromatographic analyses were performed for each point. DNA m⁵C methylase was isolated from a 0.8 M NaCl extract of nuclear pellets. Incubation was at 37° for 1 hr, as described in Materials and Methods. Triplicate assays were performed for each enzyme preparation.

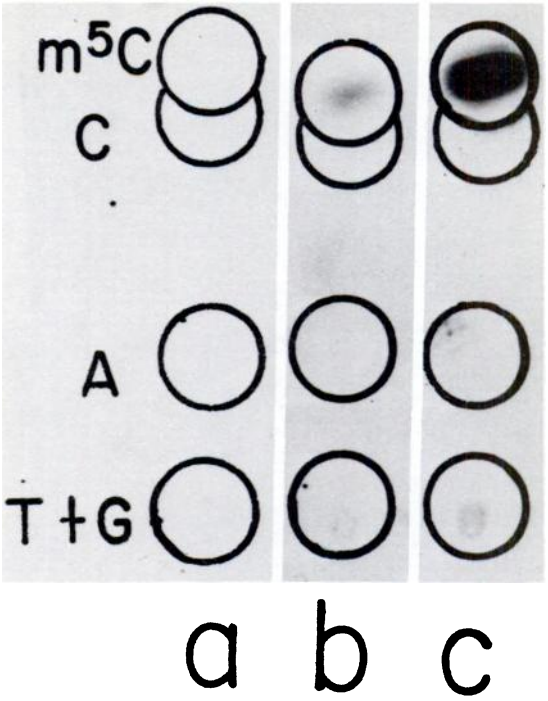

FIG. 2. *Fluorogram of one-dimensional PEI-cellulose TLC separation of deoxyribonucleoside 5′-monophosphates from in vitro methylation of homologous DNA substrates*

dpNs were obtained by digestion with nuclease P1 of DNA isolated from an in vitro DNA m⁵C methylase assay. Digest corresponding to about 100 μg of DNA was applied. Authentic dpm⁵C was co-chromatographed with the digest to locate dpm⁵C. The chromatogram was developed in methano-H_2O (1:4, v/v) to the origin, then in 0.1 N acetic acid to 19 cm above the origin, and finally in 1.0 N acetic acid to 5 cm on a Whatman No. 1 wick. dpT and dpG remained at the origin under these conditions. The methyl-deficient DNA (lane *c*) incorporated 3.1 × 10^4 cpm/50 μg of protein/60 min, while control DNA (lane *b*) incorporated 5.8 × 10^3 cpm/50 μg of protein/60 min. Lane *a* represents an assay mixture lacking substrate DNA.

ner with $0.05 < p < 0.1$ for the 4-hr time point and $p < 0.001$ for all other points studied (Student's *t* test). The same dose of 5-aza-CR inactivated DNA m⁵C methylase activity to barely detectable levels, i.e., <5% of control within 4 hr after drug treatment ($p < 0.001$), and the inhibition of enzyme activity lasted for at least 4 days. Thus, the rapid and prolonged inactivation of DNA m⁵C methylase activity appeared responsible for the decreased m⁵C synthesis in L1210 cell DNA *in vivo*.

Figure 2 represents a fluorogram of a one-dimensional separation on a PEI-cellulose layer of deoxyribonucleoside 5′-monophosphates obtained by nuclease P1 digestion of DNA isolated from the *in vitro* DNA m⁵C methylase assay mixture. In the absence of added DNA (lane *a*), no radioactivity co-chromatographed with dpm⁵C, indicating that no methyl transfer had taken place. In the presence of control DNA (lane *b*) and DNA from cells treated with 4.0 mg/kg of 5-aza-CR for 24 hr (lane *c*), ^3H radioactivity (>99%) co-chromatographed with authentic dpm⁵C. This result indicated that DNA m⁵C methylase used in this study transferred *methyl*-^3H from [CH_3-^3H]SAM to cytosine residues of added DNAs. DNAs isolated from 5-aza-CR-treated L1210 cells (lane *c*) incorporated 3.1 × 10^4 cpm/50 μg of protein/60 min. This DNA was about 5 times better as a substrate than control DNA (lane *b*), and about 2 times better than *Micrococcus luteus* DNA (result not shown). Therefore, DNA isolated from L1210 cells treated with 4.0 mg/kg of 5-aza-CR for 24 hr was used as substrate DNA for subsequent DNA methylation assays.

Mechanism of Inactivation of DNA m⁵C Methylase by 5-aza-CR Administration

Characterization of inhibitor of DNA m⁵C methylase. The kinetics of inactivation of DNA m⁵C methylase activity appeared similar to that reported by us for the inhibition of tRNA m⁵C methylase by 5-aza-CR treatment (7, 8). This suggested that DNA containing incorporated 5-aza-C, like tRNA containing incorporated 5-aza-C, would inhibit the DNA m⁵C methylase *in vivo*. Since inactivation of DNA m⁵C methylase could be detected as early as 4 hr after 5-aza-CR administration (Fig. 1), the inhibitory DNA must have been formed by this time. Therefore, DNA preparations to be tested as potential inhibitors of the methylase were isolated from L1210 cells of groups of mice treated with 0.9% NaCl, or 10.0 and 100.0 mg/kg, respectively, of 5-aza-CR for 4 hr. DNA isolated from L1210 cells exposed to 4.0 mg/kg of

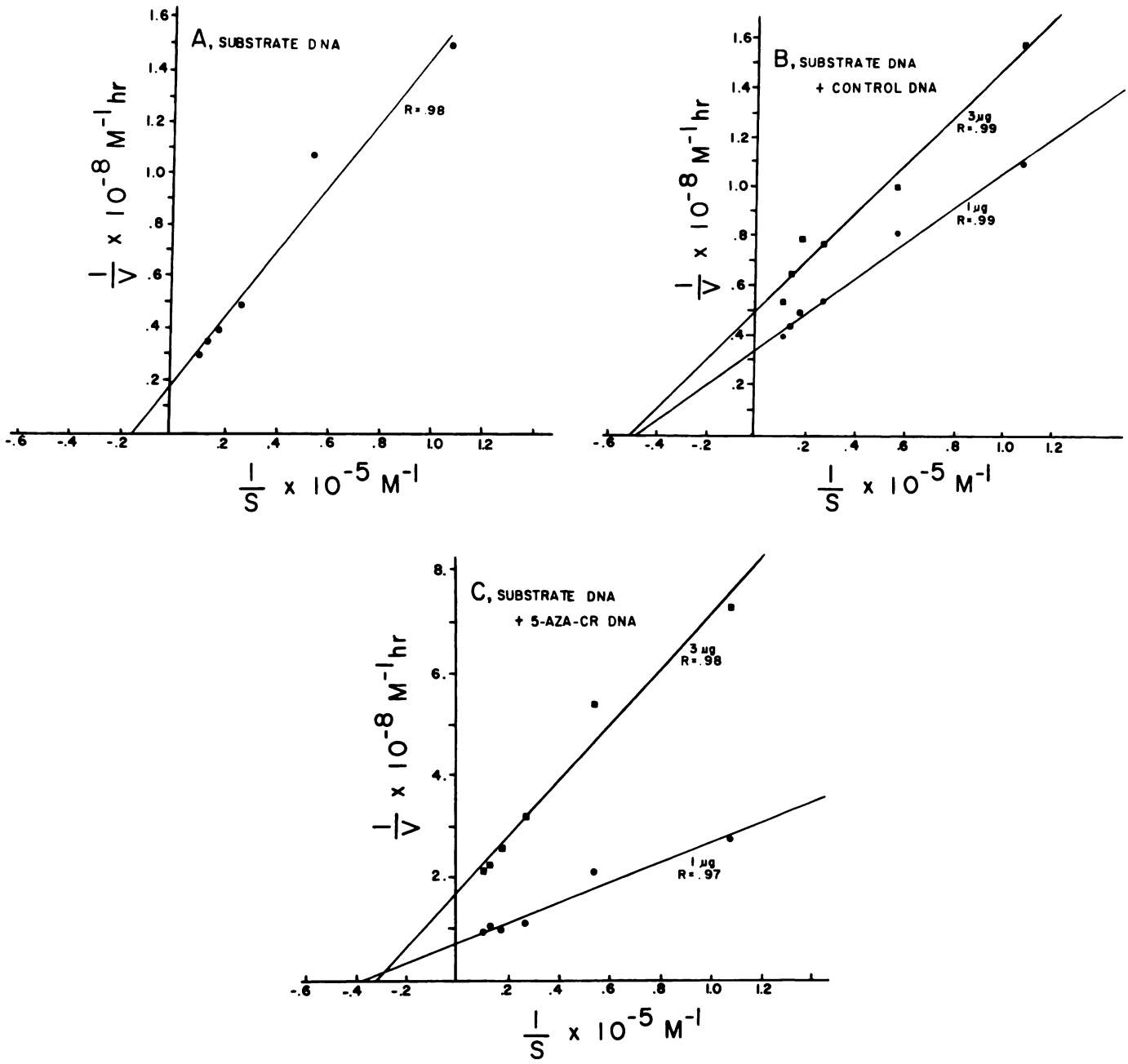

FIG. 3. *Effects of added control DNA or 5-aza-CR-modified DNA on kinetics of DNA methylation*

Double reciprocal plots show incorporation of methyl groups into DNA as a function of substrate DNA concentration at two different concentrations (1 or 3 µg) of added control DNA (from untreated L1210 cells) or 5-aza-CR-modified DNA (from L1210 cells of mice treated with 100.0 mg/kg of 5-aza-CR for 4 hr). Assay conditions were as described in Fig. 1, except that control DNA or 5-aza-CR-modified DNA was preincubated with the enzyme and [CH_3-^{3}H]SAM at 37° for 7 min. After the addition of substrate DNA, the mixture was kept at 37° for 60 min. Duplicate analyses were performed for each data point. *V*, moles of methyl groups transferred to DNA in the presence of 50 µg of protein in 100 µl of reaction mixture in 60 min. Lines were fitted to the data points by the least squares method. *R*, correlation coefficient. A, substrate DNA alone; B, substrate DNA with addition of control DNA; C, substrate DNA with addition of 5-aza-C-containing DNA. Note change of scale in panel C.

5-aza-CR for 24 hr was used as the substrate, because this was an approximately 2.8 times better substrate than DNA isolated 4 hr after drug administration. This preparation incorporated 64.8 pmol of CH_3/mg of protein/hr. On the other hand, none of the test DNA preparations served as efficient substrates (incorporating <9 pmol of CH_3/mg of protein/hr), for control enzyme. The rate of methyl transfer from SAM to the cytosine residues of substrate DNA by DNA m^{5}C methylase from control L1210 cells in the presence of test DNAs was measured. Simultaneous incubation of test DNA (25 µg) (100.0 mg/kg of 5-aza-CR) with substrate DNA (25 µg) and a limiting amount of methylase inhibited DNA m^{5}C synthesis by 50%. Addition of control DNA resulted in a 20% decrease in methylation. Preincubation of enzyme (50 µg) with test DNAs (5 µg), isolated from cells exposed

TABLE 2

The calculated V_{max} and K_m values for methylation of substrate DNA in the presence of fixed added DNA concentrations

Experimental conditions were as described in the legend of Fig. 3. Data were calculated from plots shown in Fig. 3

Added DNA		V_{max}		K_m	
Type	Amount				
	μg	nM hr^{-1}	% control	μM	% control
		52	100.0	67	100.0
Control DNA	1	29	56	21	31
	3	20	38	20	29
5-aza-CR-DNA	1	14	27	27	41.0
	3	6	11	32	47

to either 0.9% NaCl, or 10.0 and 100.0 mg/kg of 5-aza-CR, respectively, for 4 hr, and [*methyl*-^{3}H]SAM (5 μM) at 37° for 5 min before the addition of excess substrate DNA (25 μg) decreased the rate of substrate DNA methylation in a dose-dependent manner by 25.5 ± 2.8% ($p < 0.001$), 45.6 ± 3.7% ($p < 0.001$), and 63.5 ± 2.2% ($p < 0.001$), respectively. Together these experiments showed that an inhibitor of DNA m^{5}C methylase was present in the test DNA samples, and that the inhibitory effect was enhanced by preincubation of the test DNA preparations with enzyme. 5-Aza-CR has been shown to be incorporated into DNA of many different cells including L1210 cells (5, 10, 14). The dose-dependent inhibitory effects shown here suggested that the test DNAs contained different amounts of analog.

Kinetics of effects of inhibitory DNAs on enzymatic DNA methylation. To explore the nature of interaction of inhibitory DNAs with DNA m^{5}C methylase further, effects of varying the test DNA concentrations on the kinetics of enzymatic DNA methylation were studied. Figure 3A shows a double reciprocal plot of incorporation of methyl groups into substrate DNA as a function of DNA concentration without added DNA. Figures 3B and 3C show double reciprocal plots of incorporation of methyl groups into DNA as a function of substrate DNA concentration at fixed concentrations (1 and 3 μg/100 μl) of added test DNA from cells of mice treated with 0.9% NaCl (panel B) or 100.0 mg/kg of 5-aza-CR (panel C). The calculated V_{max} and K_m values have been presented in Table 2. Both test DNA preparations inhibited the V_{max} for substrate DNA methylation in a concentration-dependent manner, but the analogue-modified DNA appeared to be more effective in this respect than the untreated DNA. The changes in the K_m values of substrate DNA induced by the addition of the inhibitory DNAs were not concentration-dependent (Table 2).

Effects of 5-aza-CR Treatment on DNA Methylation of Mouse Livers

Since 5-aza-CR markedly inhibited DNA methylation of L1210 cells *in vivo*, we wondered whether this would also occur in normal tissue, such as liver. The drug is known to be readily taken up by this organ (32), leading to inhibition of tRNA m^{5}C synthesis (6). Treatment of normal mice with 7 mg/kg of 5-aza-CR once a day for 4 days had no effect on liver DNA methylation (100.6% of untreated control, $0.4 < p < 0.5$). This result was not unexpected, because little DNA synthesis takes place in normal liver. Therefore, the analogue was not appreciably incorporated into DNA, and no undermethylated DNA was synthesized. We then studied if 5-aza-CR treatment would affect DNA methylation in dividing cells of regenerating livers. As shown in Table 3, treatment with the analogue (experiment I) led to a dose-dependent decrease in DNA methylation of regenerating mouse livers. However, the decreases were variable at doses >4.0 mg/kg (experiment II), probably because of inhibition of DNA synthesis.

Since 5-aza-CR treatment did cause DNA hypomethylation in normal dividing cells, attempts were made to study the persistence of this phenomenon. As shown in Table 3, the DNA m^{5}C content of regenerating mouse liver, after treatment with 4.0 mg/kg of the drug once a day for 4 days, was 88% of control ($p < 0.001$) 7 days and 94% of control ($p < 0.001$) 14 days after partial hepatectomy, with a slight recovery at 14 days compared to 7 days ($p < 0.02$). Normal untreated regenerating liver DNA was 100% methylated 7 and 14 days after partial hepatectomy. Attempts to decrease methylation further with higher doses of 5-aza-CR and to investigate the persistence of this phenomenon in mouse liver were not successful because of the toxicity of the drug.

Long Term Observation of 5-aza-CR-Induced DNA Hypomethylation in Vivo

It has been suggested that methylation patterns might be transmitted to daughter cells by a "maintenance DNA methylase," an enzyme that would locate the methylated sites on parental DNA strands and methylate the corresponding sites on newly replicated daughter strands (31). The methylation pattern would thus be inherited by progeny cells and would normally remain invariant. 5-Aza-CR treatment induced DNA hypomethylation in

TABLE 3

Effects of 5-aza-CR treatment on DNA m^{5}C content of regenerating mouse livers

Male BALB/c Crgl mice were treated with varying doses of 5-aza-CR, beginning 24 hr after partial hepatectomy. The m^{5}C content of DNA on the days indicated in column 3 was analyzed by HPLC. At least two acid hydrolyses and four chromatographic analyses were performed. The m^{5}C content was expressed as percentage of control. Control mouse liver DNA contained 0.803 ± 0.043 mol % m^{5}C.

Experiment number	Treatment schedule days × dose	Days after partial hepatectomy	m^{5}C content[a]
	mg/kg		% control
I	4 × 0.5	7	100
	4 × 1.0	7	95
	4 × 4.0	7	88[b]
	4 × 4.0	14	94[b]
II	4 × 7.0	5	85[b]
	4 × 7.0	7	91[b]
	3 × 7.0	4	87[b]
	1 × 35.0	2	97
	2 × 35.0[c]	7	90[b]

[a] All data are derived from at least four chromatographic analyses.
[b] $p < 0.05$ (compared to control).
[c] 5-Aza-CR was given every other day.

both L1210 cells and regenerating mouse liver. It was of interest to study the long term persistence of this deficiency *in vivo*. To this end, the time course of drug-induced DNA hypomethylation in L1210 cells grown intraperitoneally was studied. The cells were transplanted periodically to maintain the cell lines *in vivo*. This technique allowed us to examine the state of DNA methylation *in vivo* for prolonged periods of time.

DNA methylation after short term treatment. Since the drug affects DNA methylation only if given during the S-phase of the cell cycle (25), the analogue was administered to mice bearing L1210 cells once a day for 4 days to ensure that all the cells would contain methyl-deficient DNA. Chronic treatment of mice with doses higher than 2.0 mg/kg resulted in severe cytotoxicity to L1210 cells (data not shown), while 1.0 mg/kg or less was tolerated.

Figure 4 shows the effect of four treatments with 1.0 mg/kg of 5-aza-CR on DNA methylation in L1210 cells. A steep increase in m^5C content of L1210 cell DNA occurred 1 week after drug withdrawal. At 7 weeks, the DNA m^5C content had recovered to a nearly normal value ($0.1 < p < 0.2$). A virtually identical pattern of recovery was observed after four doses of 0.5 mg/kg of 5-aza-CR (data not shown). To test if recovery of DNA methylation was due to selection of drug-resistant cell lines, L1210 cells obtained 13 weeks after drug withdrawal, i.e., after full restoration of DNA methylation (Fig. 4), were treated with a single dose of 4.0 mg/kg of 5-aza-CR. After 24 hr, their DNA m^5C content was found to be decreased to 71.5% of control, a value similar to that observed for identically treated control L1210 cells (data not shown), suggesting that the previously exposed cells were still sensitive to 5-aza-CR treatment.

DNA methylation after long term treatment. Since the dosing schedule described in the previous section reduced DNA m^5C content of L1210 cells by only 30%, it was of interest to determine if more prolonged treatment would decrease m^5C content further and perhaps lead to more persistent DNA hypomethylation. To this end, L1210 cells were exposed to 5-aza-CR for up to 14 treatment cycles (Table 1). The data shown in Fig. 5 indicated that prolonged treatment of leukemic cells with the analogue did not generally increase the extent of DNA hypomethylation, but the time course of recovery of DNA methylation was modified. The m^5C content of DNA of treated L1210 cells fluctuated between 50 and 70% of control during the first 10 treatment cycles (Fig. 5A–C). Continuous treatment with 5-aza-CR with gradually increasing doses (from 0.5 to 100.0 mg/kg) over a period of 35 weeks was necessary to decrease DNA m^5C content of L1210 cells to 40% of control (data not shown).

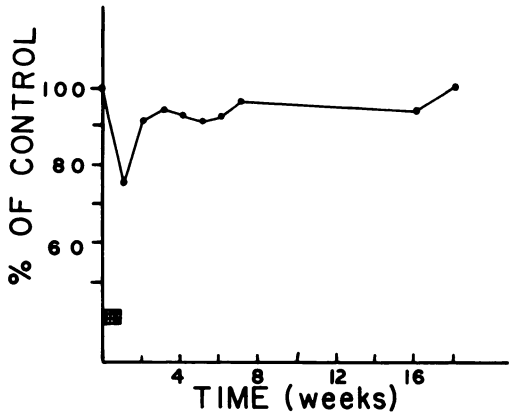

FIG. 4. *Effect of short term treatment with 5-aza-CR on L1210 cell DNA methylation level*

Male DBA-2 mice bearing L1210 cells intraperitoneally were treated with 5-aza-CR (1.0 mg/kg) once a day for 4 days, beginning 3 days after transplantation. Two days later, cells were harvested and retransplanted (1×10^5 cells/mouse) to another group of male DBA-2 mice without further drug treatment, and thereafter retransplantation was carried out once every week. The m^5C content of L1210 cell DNA was analyzed by HPLC. At least two acid hydrolyses and four chromatographic analyses were carried out for each sample. ■, period of 5-aza-CR treatment.

After prolonged exposure of L1210 cells to 5-aza-CR, initially methylation levels recovered gradually (Fig. 5). This recovery was either complete (Fig. 5A) or partial (Fig. 5B and 5C), but slower than that shown in Fig. 4. Partial recovery of methylation also occurred during chronic drug exposure (Fig. 5C and 5D). Partial or complete recovery of methylation was transient. The data shown in Fig. 5 indicate a subsequent prolonged decrease or fluctuation of DNA methylation levels. DNA m^5C methylase activities were monitored at various time points after drug withdrawal in the experiment illustrated in Fig. 5A to determine whether persistent hypomethylation was due to altered DNA m^5C methylase levels. DNA m^5C methylase activities after drug withdrawal were found to be 120% of control at 2 weeks after drug withdrawal, 105% at 5 weeks, 130% at 10 weeks, 105% at 13 weeks, and 95% at 17 weeks. Therefore, DNA hypomethylation was maintained irrespective of approximately normal DNA methylase levels.

Do cells exhibiting different DNA methylation levels grow differentially? L1210 cells containing hypomethylated DNA partially restored their methylation levels after drug withdrawal (Fig. 5). Since not all cells exposed to the drug are expected to contain equally methyl-deficient DNA, it is possible that those cells that contain highly methyl-deficient DNA might be at a growth disadvantage as compared to their counterparts containing less methyl-deficient DNA. Experiments were done to explore this possibility. To this end, control L1210 cells containing normally methylated DNA were mixed with cells containing methyl-deficient DNA. The mixed cells were retransplanted to another group of mice. Their m^5C content was determined weekly after retransplantation and compared with that of unmixed cells. Deficient L1210 cells containing 0.562 ± 0.042 mol % m^5C (unmixed cells; Fig. 6A) in their DNA were mixed with control cells containing 0.803 ± 0.043 mol % m^5C in a ratio of 2:1. The calculated m^5C content of the mixed cells was 0.642 mol % and the found m^5C content (as shown in Fig. 6A) was 0.692 ± 0.020 mol % at the time of mixing. Therefore, the ratio of the m^5C content of unmixed cells over that of mixed cells was estimated to be $0.562/0.692 = 0.81$ at the time of mixing. One week later, the DNA of the mixed cell population had 0.841 ± 0.006 mol % m^5C (104.7% of control) while the corresponding value for the unmixed cells was 0.637 ± 0.034

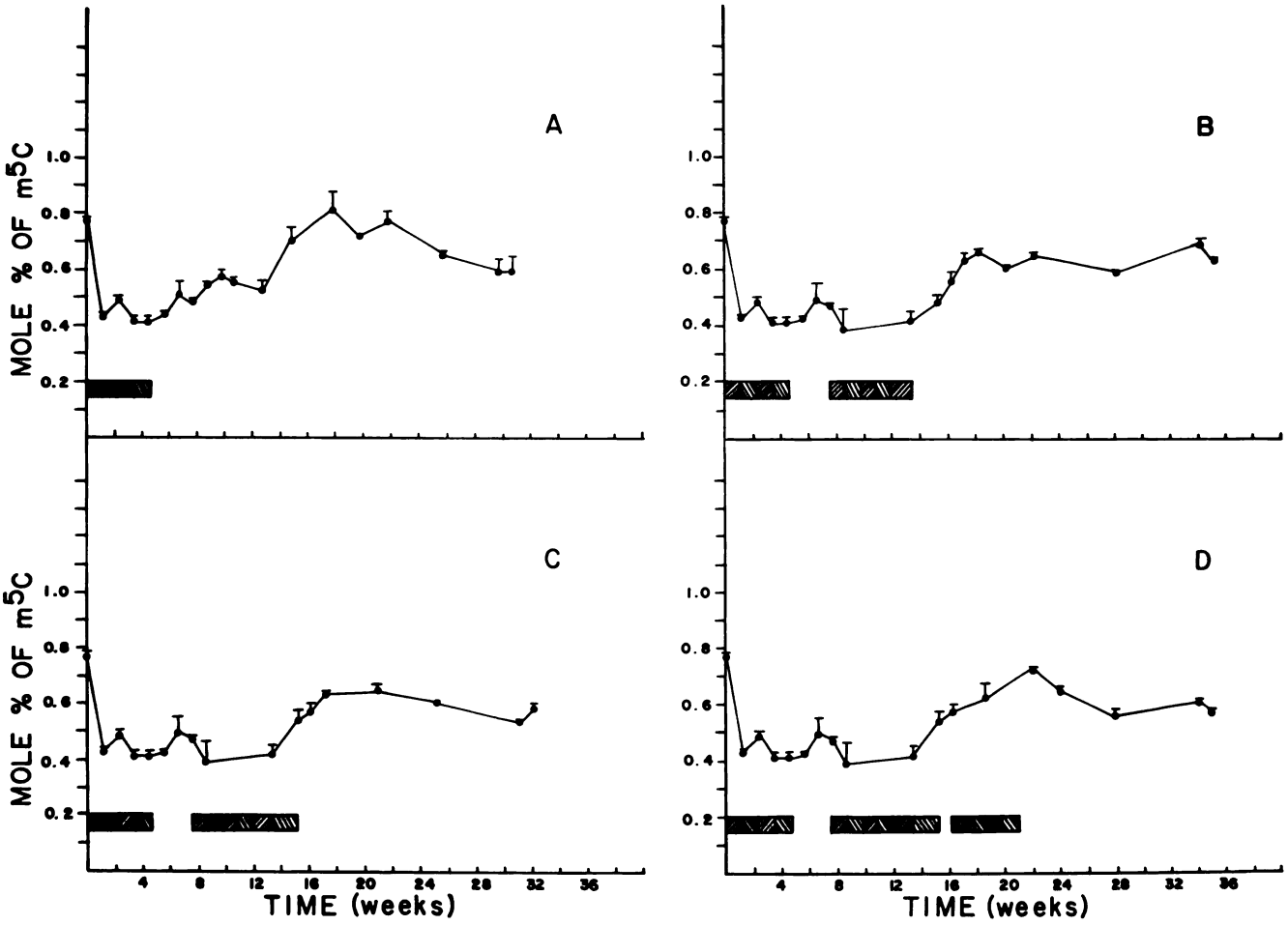

FIG. 5. *Effects of chronic treatment with 5-aza-CR on L1210 cell DNA methylation level in vivo*

Male DBA-2 mice were treated with 5-aza-CR (0.5–1.0 mg/kg) for varying lengths of time prior to drug withdrawal: panel A, four cycles of treatment with 5-aza-CR; panel B, nine cycles; panel C, 10 cycles; and panel D, 14 cycles. For treatment schedules, consult Materials and Methods (Table 1). DNA m^5C content was analyzed by HPLC. Two acid hydrolyses and at least four chromatographic analyses were performed for each sample. ▨, period of 5-aza-CR treatment. Note that the duration of each treatment cycle varied between 7 and 11 days.

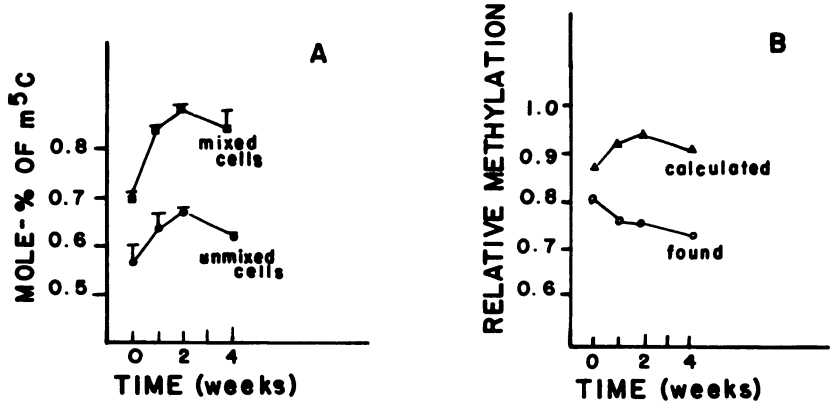

FIG. 6. *Effects of co-culturing of m^5C-deficient and control L1210 cells on DNA methylation*

L1210 cells obtained 3 weeks after drug withdrawal (*unmixed cells*; for treatment schedule, see Fig. 5B) were mixed with control L1210 cells at a ratio of 2:1 (*mixed cells*). The mixed cells were retransplanted to fresh groups of mice once a week. Their DNA m^5C content was determined by HPLC. A, mole % of m^5C; B, relative methylation = m^5C in DNA from unmixed cells/m^5C in DNA from mixed cells; calculated values = m^5C content of unmixed cells (panel A)/(m^5C content of unmixed cells × ⅔ + m^5C content of control cells × ⅓); found values, m^5C content of unmixed cells/m^5C content of mixed cells. m^5C content of control cells was 0.803 ± 0.043 mol %.

mol %. Therefore, if both control and the methyl-deficient cells doubled at the same rate, the expected m^5C ratio of unmixed cells over mixed cells would have been $0.637/(0.637 \times \frac{2}{3} + 0.803 \times \frac{1}{3}) = 0.92$ (Fig. 6B), 1 week after mixing. However, a ratio of 0.76 was found as shown in Fig. 6B. This ratio also remained lower than expected 2 and 4 weeks after mixing (panel B), suggesting that control L1210 cells survived better than their methyl-deficient counterparts.

DISCUSSION

Data presented here show that treatment of dividing cells from either neoplastic or normal tissues with 5-aza-CR *in vivo* resulted in marked inhibition of m^5C synthesis in DNA. This result was in line with the *in vitro* data reported for several lines of cultured cells (10, 14, 24, 25), for *E. coli* K12 cells (9), and for Ehrlich ascites cells (13). The drug treatment was found to have no effect on DNA methylation of resting, nondividing cells such as normal liver, strongly suggesting that DNA synthesis is a prerequisite for 5-aza-CR to exert its effect on DNA methylation. The observation of Creusot *et al.* (14) that pretreatment of cultured cells with an inhibitor of DNA synthesis, i.e., hydroxyurea, blocks the analogue-induced DNA hypomethylation agrees with data presented here.

The isolation, soon after drug treatment, of a DNA capable of decreasing the V_{max} of DNA m^5C methylase in a concentration- and dose-dependent manner *in vitro* suggested that the inhibitory DNA contained 5-aza-C and bound noncompetitively to the methylase. A similar inhibitory effect on DNA m^5C methylase of *E. coli* K12 by 5-aza-C-modified *E. coli* K12 DNA has been reported by Friedman (15), and Taylor and Jones (33) showed that a substrate DNA, isolated late from transformed mouse embryo cells after analogue treatment, inhibited DNA m^5C methylase only if preincubated with the enzyme. As shown in the present paper, careful examination of the time course of inactivation of DNA m^5C methylase activity by 5-aza-CR treatment (Fig. 1) enabled us to isolate an inhibitory DNA that was capable of inactivating DNA m^5C methylase *in vitro* without preincubation with the enzyme, but such preincubation resulted in a more extensive inhibition of the enzyme.

In addition, we have observed that DNA preparations isolated from control L1210 cells also elicited a concentration-dependent decrease of V_{max} of DNA methylation (Table 2). The reason for this effect was not clear. It was conceivably related to the mechanism of DNA methylation according to which the methylase walks along the substrate, scanning all possible methylation sites before detaching from it (34). Thus, preincubation or simultaneous incubation with control DNA would lead to a decrease in active enzyme.

Recent evidence has suggested that patterns of DNA methylation are heritable so that methylation defects should be transmitted to progeny cells (16, 31, 35, 36). Data shown in Fig. 5B and 5C are in line with this hypothesis. However, the transient, mostly incomplete restoration of methylation levels seen in Fig. 5A–D showed that only a fraction (estimated to be <40%) of unmethylated sites persisted *in vivo*. The methylation levels achieved during this phase were usually not stable. Adams *et al.* (37) reported that clones exhibited stable undermethylation after treatment of L929 cells with 5-azadeoxycytidine. The difference between these and our observations may be due to the different cell types or growth conditions (i.e., *in vivo* versus *in vitro*).

The recovery of methylation may have been due to any of several mechanisms: (i) remethylation of hemimethylated DNA by DNA methylase soon after drug withdrawal probably accounted for part of the early recoveries seen in Fig. 5A–D, as well as in Fig. 4; (ii) *de novo* remethylation of symmetrically unmethylated sites, a process for which suggestive evidence exists (38), might have taken place; (iii) selection of more viable cells, which contained more m^5C in their DNA, over less viable ones, which contained less m^5C, might have occurred, as suggested by the experiment shown in Fig. 6; (iv) the fact that DNA methylation increased during chronic drug exposure (Fig. 5C and 5D) was possibly due to development of drug-resistant cells, whose DNA became remethylated.

The mostly incomplete restoration of DNA methylation seen in Fig. 5 was accompanied by a decrease of DNA methylation levels upon retransplantation. This may have been due to different mechanisms. (i) Demethylation may have occurred. The literature gives examples for loss of methyl groups of foreign DNA introduced by transfection into mammalian transformed cells (35, 36). In addition, a DNA-demethylating activity has been detected in nuclei of murine erythroleukemia cells (39). (ii) A continuous selection of clones having different extents of DNA methylation may have conceivably given rise to clones which were highly viable in spite of their low overall extent of methylation. In accord with this, tumor DNAs have been reported to exhibit a considerable degree of undermethylation (16, 26, 27).

Any chemotherapeutic agent or chemical causing more or less random structural alterations of DNA in individual cells, for example, by covalent binding, analogue incorporation, or inhibition of methylation, is expected to convert a genetically homogeneous population of normal or transformed cells into a heterogeneous one, because the genome of each cell within the exposed population is altered in a unique way. Subsequent selection coupled with demethylation or remethylation would then lead to long-term instability of DNA methylation levels, as reported here. Such instability of DNA methylation may be involved in the genesis of tumor cell heterogeneity, as proposed by Frost and Kerbel (40).

In conclusion, the mechanism of inhibition of DNA m^5C methylase by 5-aza-CR *in vivo* appears identical to the one previously defined for tRNA m^5C methylase: soon after drug administration an inhibitory nucleic acid containing incorporated analogue binds to the respective methylase, thereby inactivating the enzyme, and leading to the biosynthesis of m^5C-deficient DNA and tRNA, respectively. The methyl deficient DNA was found to show a complex pattern of recovery of methylation after prolonged drug treatment of L1210 cells: initial partial recovery was followed by either a prolonged period of decline or fluctuation of the m^5C levels. Thus, while

DNA hypomethylation of a transformed cell population can be partially maintained *in vivo*, the methylation defect does not necessarily remain constant but may be disturbed under certain conditions, giving rise to long term instability of DNA methylation patterns in progeny cells.

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Send reprint requests to: L.-J. W. Lu, Department of Pharmacology, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030.