Effect of 5-Azacytidine on Differentiation and DNA Methylation in Human Promyelocytic Leukemia Cells (HL-60)

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ABSTRACT

One of the most readily quantitated indices of myeloid maturation in HL-60 cells is their ability to respond to the tumor promotor, 12-O-tetradecanoylphorbol-13-acetate with increased respiratory burst activity. HL-60 cells exposed to the antileukemic drug, 5-azacytidine (3 to 5 μM) for 24 hr and subsequently cultured in its absence for 2 to 3 days develop an enhanced ability to respond to 12-O-tetradecanoylphorbol-13-acetate with increased respiratory burst activity detectable as an increase both in hexose monophosphate shunt activity and in the proportion of the population producing superoxide anion. 5-Azacytidine treatment also causes marked inhibition of DNA methyltransferase, and thus DNA synthesized by HL-60 cells during the 24-hr period of analogue treatment is essentially devoid of methylated cytosine residues. This suggests, as does our previous finding that a general inhibitor of transmethylation reactions, L-ethionine, can induce differentiation of HL-60 cells, that changes in gene expression triggered by these compounds may be linked to their ability to alter patterns of DNA methylation. Since at least 50% of HL-60 cells capable of forming colonies in soft agar after a 24-hr exposure to 5-azacytidine yield progeny that mature (i.e., produce superoxide anion in response to 12-O-tetradecanoylphorbol-13-acetate) 2 weeks after 5-azacytidine treatment, the results also indicate that the changes induced in HL-60 cells by limited exposure to 5-azacytidine are heritable and can influence gene expression many generations after treatment has been terminated.

INTRODUCTION

5-aza-Cyd, a cytidine analogue in which ring carbon 5 is replaced with nitrogen, is currently used in treatment of acute myelogenous leukemia (32). Its effects on cellular metabolism, both in vivo and in vitro, are varied and include mutagenesis, induction of chromosome breakage, interference with processing of ribosomal RNA, and an inhibition of synthesis of DNA, RNA, and protein (see Ref. 6 for review). 5-aza-Cyd is also incorporated into RNA and DNA of prokaryotic and eukaryotic cells (19, 30). Several lines of evidence indicate that, as a consequence of this incorporation, methylation of cytosine residues in both forms of nucleic acid is inhibited (11, 15, 18, 20).

Recently, a number of groups, including our own, have reported that 5-aza-Cyd is capable of inducing differentiation or altered gene expression in cultured cells (3, 7, 9-11, 17, 18, 24, 26, 28). In many of these studies, it was further demonstrated that 5-aza-Cyd treatment resulted in the appearance of hypomethylated sites in DNA (7, 9, 11, 17, 18, 26, 28). It has been rather generally assumed that the DNA in treated cells becomes hypomethylated because 5-aza-Cyt residues in DNA cannot accept methyl groups. However, we have shown that DNA methyltransferase activity is rapidly lost when mouse cells (Friend erythroleukemia cells) are exposed to 5-aza-Cyd and 5-aza-dCyd and that this loss requires incorporation of 5-aza-Cyt into DNA. Our data indicate (a) that hypomethylation of DNA occurs in treated cells because DNA synthesis continues in the absence of sufficient active DNA methyltransferase, and (b) that DNA methyltransferase is directly inhibited by its interaction with 5-aza-Cyt-substituted DNA (4, 11).

Here, we describe the effects of 5-aza-Cyd treatment on cells of human origin, a line (HL-60) derived from the peripheral blood of a patient with acute promyelocytic leukemia (8). We have examined the response of these cells to 5-aza-Cyd. (a) Since all of the reported experiments demonstrating both 5-aza-Cyd-mediated alteration of gene expression and accompanying loss of methyl groups from DNA involved use of mouse or chicken cells and since 5-aza-Cyd treatment had been found to activate genes on the human X chromosome in mouse-human hybrid cells (26) but not in human diploid cells (33), we wished to determine whether 5-aza-Cyd could have a coordinated effect on gene expression and DNA methylation in human cells. (b) Since loss of DNA methyltransferase had not been demonstrated in 5-aza-Cyd-treated human cells, we felt that it was of some importance to determine whether or not 5-aza-Cyd-mediated inhibition of DNA methylation in human cells occurred by the same mechanism as in murine cells. (c) Since it has often been proposed that compounds which could induce malignant cells to differentiate might be of therapeutic value (16, 21, 31), we wished to determine whether 5-aza-Cyd, which is used as a chemotherapeutic agent for treating human leukemias, was a particularly effective inducer of differentiation of human leukemic cells. In this report, we present evidence that 5-aza-Cyd treatment triggers granulocytoid differentiation in HL-60 cells, that DNA methyltransferase activity is profoundly inhibited in the treated cells, and that DNA synthesized while the cells lack active DNA methyltransferase is virtually free of m5Cyt residues. However, we also find that 5-aza-Cyd is less effective in inducing differentiation of HL-60 cells than are other compounds such as dimethyl sulfoxide and L-ethionine and that, at concentrations greater than 5 μM, the cytotoxic effects of 5-aza-Cyd increase as ability to induce differentiation is decreased. A preliminary report of these studies has been presented (24).

MATERIALS AND METHODS

Reagents. Media and serum were from Grand Island Biological Co.
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(Grand Island, N. Y.), agar was from Difco Laboratories (Detroit, Mich.), and tissue culture ware was from Falcon Plastics (Oxnard, Calif.). [methyl-3H]-S-AdoMet was purchased from ICN Pharmaceuticals (Cleveland, Ohio) and [1-14C]glucose was from New England Nuclear (Boston, Mass.). TPA was from Consolidated Midland Corporation (Brewster, N. Y.) and 5-aza-Cyd was from Sigma Chemical Co. (St. Louis, Mo.). Because of its liability in aqueous solution (29), concentrated solutions of 5-aza-Cyd were prepared in glass-distilled water immediately before filter sterilization and dilution into cell cultures.

Cells. The human leukemic cell line, HL-60, was the gift of Dr. S. J. Collins, National Cancer Institute, Bethesda, Md. The cells are maintained in suspension culture in Roswell Park Memorial Institute Medium 1640 supplemented with 10% heat-inactivated FBS. For treatment with 5-aza-Cyd, the cells were seeded at 2.5 x 10^5/ml in growth media, and the indicated amounts of 5-aza-Cyd were added. For induction with dimethyl sulfoxide or L-ethionine, the cells were diluted to the same concentration in growth medium containing 1.15% dimethyl sulfoxide or 3 mM L-ethionine, respectively.

Measurement of CO2 Release. Cells were washed in 0.9% NaCl solution and resuspended at 5 x 10^6/ml in glucose-free Earle’s balanced salt solution buffered at pH 7.2 with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and supplemented with 10% dialyzed FBS and 0.2 mM of [1-14C]glucose (specific activity, 5.7 mCi/ml). TPA was added to 1.66 x 10^-8 M as indicated. CO2 released by 1 ml of cell suspension during a 1-h incubation at 37°C was trapped in a Hyamine-soaked filter paper in a glass well suspended above the incubation mixture (34). The reaction was terminated by injection of 100 μl of concentrated sulfuric acid, and incubation continued for 15 min at 37°C to ensure that all dissolved CO2 was released from the acidified medium.

Measurement of Superoxide Anion Production. The percentage of cells producing superoxide anion was determined by counting the number of cells capable of reducing NBT. Cells were suspended at 2 x 10^5/ml in Roswell Park Memorial Institute Medium 1640 supplemented with 20% FBS and incubated for 20 min with an equal volume of 0.2% NBT dissolved in 0.15 M NaCl-0.015 M sodium phosphate, pH 7.4, in the presence and absence of 1.66 x 10^-6 M freshly diluted TPA. At least 200 cells from each assay tube were counted to determine the percentage of cells containing intracellular reduced blue-black formazan deposits.

Extraction of DNA Methytransferase and Methylation of HL-60 Cell DNA in Vitro. To prepare DNA methyltransferase, 10^6 washed cells were sonicated in 5 ml of buffer 0.3% Triton X-100 (0.3 ml Triton in 100 ml of 0.32 M sucrose-10 mM Tris-HCl, pH 7.8-3 mM MgCl2-0.1 mM phenylmethylsulfonyl fluoride-0.5 mM dithiothreitol). Nuclei were pelleted by centrifugation at 1,000 x g for 5 min, washed once in the same buffer, and resuspended in 0.5 ml of 0.01 M Tris-HCl, 0.5 mM MgCl2-0.1 mM phenylmethylsulfonyl fluoride.

The nuclear suspension was quick frozen in dry ice-ethanol, thawed, brought to 0°C, and NaCl by adding 3 M NaCl, and kept at 0°C for 15 min. After disruption in a Dounce homogenizer, the suspension was clarified by a 2-hr centrifugation at 45,000 x g. The supernatant was assayed directly for DNA methyltransferase activity without further purification, since it was determined that concentrations of NaCl up to 50 mM in the standard assay mixture did not affect incorporation of methyl groups into DNA.

Comparison of methyl acceptance of DNAs isolated from HL-60 cells before and after treatment with 5-aza-Cyd, DNAs isolated by the method of Marmur (22) were incubated for 1 hr at 37°C under standard assay conditions with 16 μM [methyl-3H]-S-AdoMet and an excess of DNA methyltransferase (>8 units/5 μg DNA) isolated from FL cells (5).

Determination of the mCyt Content of HL-60 Cell DNA. Cells in the logarithmic phase of growth were incubated for 12 to 18 hr at 37°C in minimum essential medium, 10% FBS containing [6-3H]uridine (0.2 μCi/ml; specific activity, 24.2 Ci/mmol). When methylation in the presence of 5-aza-Cyd was to be measured, the analogue was added 4 hr prior to the addition of [6-3H]uridine, and incubation was continued for 18 hr. DNA was isolated from the cells by the method of Blixt and Stafford (2). Radiolabel in cytosine and m5Cyt was quantitated after chromatographing perchloric acid hydrolysates of the DNAs (minimum sample, 125,000 cpm) on a Whatman Partisil SCX column (0.4 x 25 cm) as described previously (5).

RESULTS

Effect of Exposure to 5-aza-Cyd on Growth and Differentiation of HL-60 Cells. As we have reported previously (23, 25), dimethyl sulfoxide- and L-ethionine-induced granulocytic maturation of HL-60 cells is associated with the appearance of a TPA-sensitive NADPH oxidase. Activation of this enzyme by addition of TPA to the culture medium results in an immediate and marked increase in the rate with which the cells catabolize glucose via the hexose monophosphate shunt. Thus, one can conveniently quantitate the degree to which HL-60 cells have matured along the granulocytic pathway by measuring their ability to release CO2 from C-1 of glucose in the presence of TPA. As can be seen in Table 1, 3 days after HL-60 cells are exposed to 5-aza-Cyd for 24 hr, they display a TPA-stimulated increase in hexose monophosphate shunt activity which is significantly greater than that of untreated cells. The maximal

![Table 1: Effect of 5-aza-Cyd on growth and maturation of HL-60 cells](image)
response occurs in cultures which have been exposed to 3 to 5 \( \mu M \) 5-aza-Cyd. TPA-stimulated CO\(_2\) release by cells in these cultures approaches 55% of that found for HL-60 cells grown for 4 days in the presence of 1.15% dimethyl sulfoxide, one of the most potent inducers of HL-60 cell differentiation (8), and 78% of that found for HL-60 cells grown for 4 days in the presence of L-ethionine, an inducer of differentiation (25) which, in the form S-AdoEth also acts to inhibit S-AdoMet-dependent transmethylation reactions, including DNA methylation.

The data presented in Table 1 further demonstrate that the increase in TPA-stimulated hexose monophosphate shunt activity resulting from 5-aza-Cyd is dependent on analogue concentration over the range of 0.1 to 5 \( \mu M \) and is paralleled by an increase in the percentage of cells in the population stimulated to produce superoxide anion in the presence of TPA. Morphological examination of Wright-Geimsa-stained cells from these cultures taken 4 days after exposure to 5-aza-Cyd revealed an increased percentage of mature myeloid forms, primarily metamyelocytes [20 to 25% after 5 \( \mu M \) 5-aza-Cyd compared with less than 10% in untreated cultures, and 60 to 80% in dimethyl sulfoxide (8, 23) and L-ethionine-treated cultures (24)]. These results indicate that 24 hr of exposure to 5-aza-Cyd is sufficient to trigger granulocytoid differentiation in HL-60 cells. However, whether the criteria used to define the extent to which cells in the culture have matured is morphology or ability of the cells to respond to TPA with increased hexose monophosphate shunt activity or superoxide anion production, it is clear that 5-aza-Cyd is not as efficient in inducing HL-60 cell differentiation as either dimethyl sulfoxide or L-ethionine.

Attempts to increase the fraction of mature cells by prolonging the time cells were maintained in 5-aza-Cyd-supplemented medium or by raising the initial concentration of 5-aza-Cyd above 5 \( \mu M \) were unsuccessful. Cultures maintained in 5-aza-Cyd-supplemented medium for 4 days have the same fraction of viable cells capable of responding to TPA with superoxide anion production and a growth rate only 10 to 15% slower than cultures of cells reseeded in fresh medium after a 24 hr exposure to 5-aza-Cyd (data not shown), a finding which can probably be accounted for by the instability of the analogue in neutral aqueous solution (29). However, even when the cells are washed free of 5-aza-Cyd after 24 hr, both cell viability and the fraction of treated cells maturing are markedly reduced if initial concentrations of 5-aza-Cyd exceed 5 \( \mu M \) (Table 1). Thus, it was necessary to consider the possibility that 5-aza-Cyd might be less effective than dimethyl sulfoxide in inducing differentiation because it or one of its metabolites could partially block the maturation process.

Because cells which produce superoxide anion reduce NBT even when they are embedded in soft agar, it is possible to observe the long-term effects of 5-aza-Cyd treatment on the maturation of the progeny of individual HL-60 cells and to determine how treatment affects the ability of cells to respond to dimethyl sulfoxide as an inducer of differentiation under conditions where differential growth rates of differentiating and nondifferentiating cells do not affect scoring. The studies are, however, complicated by low cloning efficiency of HL-60 cells (10 to 20% of cells plated) and a decrease in cloning efficiency (1 to 5% of cells plated) resulting from 5-aza-Cyd treatment. The data presented in Table 2 indicate that, although 5-aza-Cyd increases the percentage of cells able to produce superoxide anion to 15 to 25% of cells in the population with 4 days after treatment (NBT-positive cells; Table 1), few of these cells retain the ability to form colonies in soft agar. This loss is reflected in the virtual absence, both at 7 and 15 days, of small colonies composed completely of NBT-positive cells (Fig. 1A).

However, the colonies which do form gradually develop subpopulations of mature cells (NBT-positive and NBT-negative mixed colonies, Fig. 1, B and C), and by 13 to 15 days after treatment with 5-aza-Cyd at least one-half of the colonies (average size, 30 to 40 cells) contain 5 or more cells capable of producing superoxide anion in the presence of TPA. In contrast, fewer than 2% of colonies from untreated cultures contain NBT-positive cells. The observed lag of 1 to 2 weeks before cells with a mature phenotype appear in the colonies formed by 5-aza-Cyd-treated cells does not occur as the result of a requirement for time to recover from toxic effects of 5-aza-Cyd before differentiation can occur. The percentage of cells which become rapidly committed to differentiation in agar supplemented with dimethyl sulfoxide and form colonies in which all cells mature within 7 days is approximately the same, whether the cells have been pretreated with 5-aza-Cyd or not. Furthermore, without ameliorating the inhibitory effect of 5-aza-Cyd on colony formation, dimethyl sulfoxide increases the percentage of 5-aza-Cyd-treated cells giving rise to colonies with differentiated subpopulations. Although the 5-aza-Cyd-treated cells do not form as many mixed colonies as do untreated cells during the first 7 days of culture in dimethyl sulfoxide, dimethyl sulfoxide increases the percentage of mixed colonies present in such cultures by 4- to 5-fold. Interestingly, the highest level of mixed colonies in cultures of cells pre-treated with 5-aza-Cyd is still observed after 15 days. This proportion is (a) greater than that found with 5-aza-Cyd-treated cells in the absence of dimethyl sulfoxide, (b) exceeds that found at any time during the culture of untreated cells in the presence of dimethyl sulfoxide, and (c) occurs at a time when the proportion of mature colonies in dimethyl sulfoxide cultures of untreated cells is already diminishing, presumably as a result of lysis of cells which differentiated earlier in the culture period. These results suggest the possibility that there are 3 distinct

![Table 2](image-url)
populations among the HL-60 cells which retain the ability to form colonies after 5-aza-Cyd treatment, one population which can differentiate in the presence of dimethyl sulfoxide but which does not differentiate in its absence, a second more slowly maturing population which differentiates in response to 5-aza-Cyd, and a third which does not respond to either inducing agent (Fig. 1D).

Effect of 5-aza-Cyd on Methylation of HL-60 Cell DNA. As shown in Table 3, 5-aza-Cyd treatment has a profound effect on DNA methylation in HL-60 cells. DNA isolated from the cells 24 hr after adding 5-aza-Cyd (4 μM) to their culture medium can be methylated to a 9- to 10-fold higher level in vitro than DNA from untreated cells, indicating that it contains more potentially methylatable sites than does DNA from untreated cells. Since there are no known enzymes which remove methyl groups from DNA and since few cells would have been likely to undergo even one complete round of DNA replication in 24 hr (doubling time for HL-60 cells under standard growth conditions is 48 hr), it is probable that the majority of these sites are hemimethylated ones resulting from the inhibition of methylation of newly synthesized DNA.

Direct measurement of the extent of modification of cytosine residues in DNA synthesized during 5-aza-Cyd treatment confirms that the compound does indeed severely inhibit methylation of newly synthesized DNA. DNA of treated and untreated cells was labeled with [6-3H]uridine, which is a common precursor of both cytosine and m^5Cyt residues in DNA. Since methylation of cytosine residues occurs only after they have been incorporated into DNA, radiolabel in cytosine and m^5Cyt will be derived from the same nucleotide pool. Thus, determination of the ratio of radiolabeled m^5Cyt to that of cytosine plus m^5Cyt in DNA gives a measure of the extent to which cytosine residues are methylated in DNA synthesized during the labeling period which is unaffected by any shifts in nucleotide pool size caused by 5-aza-Cyd treatment. As shown in Table 3, approximately 3.1% of the cytosine residues incorporated into HL-60 DNA during an 18-hr labeling period are methylated. In contrast, fewer than 0.25% of cytosine residues incorporated into DNA during an 18-hr period starting 4 hr after addition of 5-aza-Cyd to the medium are methylated. Judging from the rate of incorporation of [6-3H]uridine into DNA (see footnotes to Table 3), synthesis of DNA continues in 5-aza-Cyd-treated cells at a rate 30 to 35% of that in untreated cells for at least 40 hr. However, within 4 to 8 hr after exposing the cells to 5-aza-Cyd, the amount of active DNA methyltransferase which can be extracted from cell nuclei is reduced to 15 to 20% of that extractable from nuclei of untreated cells (Table 3). This reduction in level of active enzyme persists throughout the treatment.

It should be noted that the almost complete block of DNA methylation (>95%) which results from the loss of 80 to 85% of active DNA methyltransferase indicates that DNA methylation in HL-60 cells is more sensitive to inhibition by 5-aza-CR treatment than is DNA methylation in FL cells, where an 85% loss of enzyme activity in 4 to 6 hr results in reduction of approximately 50% in the level of DNA methylation (11). High-performance liquid chromatography quantitation of radiolabeled [4-14C]5-aza-dCyd in DNA isolated from HL-60 cells exposed to 4 μM [4-14C]5-aza-Cyd for 20 hr indicates that approximately 0.5% of cytosine residues are replaced with 5-aza-Cyt during standard treatment, an incorporation of analogue well within the range found for murine cells exposed to 5-aza-Cyd (11,18). Thus, it seems unlikely that the difference in sensitivity of DNA methylation is due to a more efficient incorporation of 5-aza-Cyd into DNA by HL-60 cells as compared to FL cells. It may, however, reflect a more obvious difference between FL and HL-60 cells, i.e., that the average amount of DNA methyltransferase which can be isolated from untreated FL cells is 28 to 30 units/10^9 cells (11) as compared with 20 to 25 units/10^9 untreated HL-60 cells (Table 3). Since DNA synthesis is no more severely inhibited in 5-aza-Cyd-treated HL-60 cells than in treated FL cells, an 85% reduction in the initially lower level of DNA methyltransferase in HL-60 cells could account for the virtual absence of m^5Cyt residues in DNA synthesized by HL-60 cells during 5-aza-Cyd treatment.

### DISCUSSION

The results presented above demonstrate that 5-aza-CR can induce differentiation of HL-60 cells. It shares this property with a variety of other nucleoside analogues which inhibit enzymes involved in DNA synthesis or which can be directly incorporated into DNA in place of one of the standard nucleotides (3). Like these other compounds, 5-aza-Cyd is incorporated into DNA and eventually slows the rate of DNA synthesis. However, as shown here, in the concentration range where 5-aza-Cyd acts as an inducer of differentiation, it affects the methylation of cytosine residues in newly synthesized DNA as described in "Materials and Methods" and Ref. 11 using hypomethylated FL cell DNA as substrate.

**Table 3**

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>in vitro methyl</th>
<th>DNA methylation</th>
<th>m^5Cyt</th>
<th>Cytosine + m^5Cyt</th>
<th>m^5Cyt</th>
<th>× 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-aza-Cyd 100 ± 15°</td>
<td>4 ± 0.5° (4 hr)</td>
<td>0.24 (4-22 hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None 11 ± 4</td>
<td>20 ± 1.5</td>
<td>0.14</td>
<td>3.1</td>
<td>0.14</td>
<td>3.0 ± 0.15</td>
<td></td>
</tr>
</tbody>
</table>

- Determined as described in "Materials and Methods" and Ref. 11 using hypomethylated FL cell DNA as substrate.
- Specific activity of DNAs used in these experiments were 25 ± 3 x 10^2 cpm/mg for DNAs extracted from untreated HL-60 cells radiolabeled for 18 hr with [H]-uridine in the logarithmic stage of growth: 9 ± 1.3 x 10^2 cpm/mg/hr for DNAs extracted from untreated HL-60 cells radiolabeled with [H]-uridine starting at the fourth hr of a 22-hr treatment with 4 μM 5-aza-Cyd and 7.8 ± 2 x 10^2 cpm/mg for DNAs from HL-60 cells radiolabeled for 18 hr starting 24 hr after initiation of 5-aza-Cyd treatment.
- Average ± S.D. of 3 determinations on 4 separate DNA preparations using a 0.3 M NaCl extract from FL cell nuclei as source of DNA methyltransferase (11).
- Average ± S.D. of duplicate determinations in 3 experiments taken from the range of linear increase of incorporation in response to increased enzyme concentration. The amount of enzyme extractable from nuclei of untreated cells was the same at 4 and 18 hr.
- No radiolabeled material could be detected coeluting with m^5Cyt when acid hydrolysates of the DNAs were analyzed by high-performance liquid chromatography, as described in "Materials and Methods." Under conditions where 1% methylation of cytosine residues would be expected to yield 3000 cpm of m^5Cyt. The value for m^5Cyt content of DNA synthesized during the first 24 hr of 5-aza-Cyd treatment is at the maximum value rather than the average observed (4 samples).

5 The following possible reasons for the difference in levels of DNA methyltransferase which can be extracted from FL and HL-60 cells before and after 5-aza-Cyd treatment have been ruled out: differences in the extent of leakage of inhibitors of DNA methylation in enzyme extracts.
much more rapidly and to a greater extent than it affects the rate of synthesis of DNA.

It is evident that HL-60 cells exposed to 5-aza-Cyd suffer a loss of at least 80% of DNA methyltransferase activity within 4 hr and that DNA synthesized subsequent to this is methylated to less than 5% of the normal level. This finding indicates that 5-aza-Cyd treatment affects DNA methylation by mediating the inhibition of DNA methyltransferase in both human and mouse leukemia cells. It also argues against a mechanism of DNA demethylation based solely on the inability of 5-aza-Cyt residues in DNA to accept methyl groups since the level of substitution of 5-aza-Cyt in DNA of both human and murine cells exposed to 1 to 10 μM 5-aza-Cyd for 24 hr is between 0.3 and 5% of cytosine residues and only 3 to 4% of these cytosine residues are in methylation sites ("Results", Refs. 11 and 18).

Even though our studies of the effects of 5-aza-Cyd treatment on HL-60 cells indicate that the process of DNA methylation in human leukemia cells can be highly sensitive to inhibition by this drug, they also indicate that it is highly unlikely that the differentiation which accompanies loss of methyl groups from DNA makes a major contribution to the therapeutic value of 5-aza-Cyd in treating human leukemias. While 5-aza-Cyd can act to induce differentiation of HL-60 cells at noncytotoxic concentrations, its relatively low efficiency as an inducer (10 to 15% of cells to survive and to retain growth potential. However, at concentrations of 5-aza-Cyd no more than double those which are maximal for inducing differentiation, a 24-hr exposure of HL-60 cells to the drug reduces cell viability by more than 60% in the absence of any increase in the percentage of cells that mature.

Although we cannot rule out the possibility that human leukemia cells in situ are more readily induced to differentiate by 5-aza-Cyd than are HL-60 cells, a number of reports in the literature point out the fact that inefficient activation of gene expression by 5-aza-Cyd is not unique to HL-60 cells. Approximately 15% of 5-aza-Cyd-treated FL cells go on to accumulate hemoglobin (11), fewer than 15% of treated C3H 10T1/2 cells go on to form myotubes (10), and in some systems activation of specific selected genes occurs in fewer than 20 of 10⁴ cells (7, 28).

This leaves us with question as to the relationship between the effects of 5-aza-Cyd on DNA methylation and its effects on differentiation. Because 5-aza-Cyd acts as an inducing agent in the same concentration range where it mediates loss of active DNA methyltransferase from cells and because 5-aza-Cyd must be incorporated into DNA both to trigger differentiation (9, 17) and to act as an inhibitor of DNA methylation (4, 11), it is tempting to postulate that the 2 events are more than coincidentally linked. This is particularly true in light of the accumulating evidence indicating that gene expression can be inhibited by methylation of cytosine residues in DNA and because of the general association between either total absence of m⁵Cyt residues or absence of m⁵Cyt residues in specific sites with active gene expression (12, 13). However, even if it is accepted that alterations in gene expression in 5-aza-Cyd-treated cells are a result of loss of methyl groups from DNA, it must still be explained why such a profound drop in the level of DNA methylation as is observed in HL-60, FL (11), and C3H10T1/2 cells (18) triggers differentiation in so few cells.

It has been suggested that failure of 5-aza-Cyd to activate genes might indicate that gene activation requires a more extensive demethylation than can be achieved with nontoxic levels of the compound (14). Our results support this suggestion, in the sense that we find that limited exposure (24 hr) to 5-aza-Cyd at concentrations greater than 5 μM does not trigger differentiation of HL-60 cells but leads to decreased cell viability and a reduced rate of growth. However, at concentrations where maximal differentiation is observed, the failure of 70 to 80% of 5-aza-Cyd-treated cells to differentiate despite a virtually complete block of methylation of newly synthesized DNA cannot be ascribed to either limited demethylation or toxicity.

The results reported here for HL-60 cells and our previous findings with FL cells (11) demonstrate that, under conditions of treatment with 5-aza-Cyd that trigger maximal differentiation (10 to 20% of the population), the treated cells which do not respond to 5-aza-Cyd are still capable of differentiating in response to another inducer, dimethyl sulfoxide.

An alternative explanation, suggested by our observation that hemimethylated sites in DNA synthesized during 5-aza-Cyd treatment can be "remethylated" once cells recover DNA methyltransferase activity (4, 11), is that only a limited number of hemimethylated sites actually remain unmethylated long enough to allow another full round of DNA synthesis and establishment of completely unmethylated sites in critical regulatory regions. The efficiency of 5-aza-Cyd as an inducer, as well as the observed specificity with which it acts to induce characteristic changes in gene expression in different cell types, would then rely on the existence of factors (DNA-binding proteins or conformational alterations) which could act to block or reduce the rate of methylation of hemimethylated sites in specific DNA regions. If there is a similar requirement for such factors during normal development, cultures of cells such as HL-60 or FL which continually generate small populations of differentiated cells might be expected to contain subpopulations of cells which already contain these factors and perhaps even of cells with an increased frequency of hemimethylated sites associated with genes "next on the program" to be activated. The developmental fate of the cells after 5-aza-Cyd treatment would then be expected to vary as a function of (a) whether they already contain hemimethylated sites in "critical" regions, (b) the intracellular concentration of factors which stabilize hemimethylated sites, and (c) the rate at which normal levels of active DNA methyltransferase are restored, and might account for the finding that some HL-60 cells can express differentiated functions 2 to 3 days (one cell division) after the process of DNA methylation is perturbed by 5-aza-Cyd, while others require 1 to 2 weeks. However, until critical methylation sites have been defined and until it is understood how DNA methylation is regulated, this explanation can be regarded only as speculation.

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Fig. 1. Typical colonies formed by 5-aza-Cyd-treated HL-60 cells growing in soft agar. Cells capable of responding to TPA with production of superoxide anion are dark with deposits of insoluble blue-black formazan (NBT-positive). A, small colony composed completely of NBT-positive cells. Note: some cells are more darkly stained than others. B and C, colonies composed of a mixture of NBT-positive and NBT-negative cells. D, a large NBT-negative colony. Photomicrographs of colonies tested for NBT dye reduction 14 days after plating in soft agar. Details of culture and staining are described in Table 2. × 200.
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