Inhibition of Dimethyl Sulfoxide Induced Erythropoietic Differentiation of Murine Erythroleukemia Cells in Culture

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ABSTRACT

The dimethyl sulfoxide induced erythropoietic differentiation of murine erythroleukemia cells, as determined by scoring benzidine positive cells, is inhibited by mitomycin C at concentrations that have no effect on cell proliferation. The inhibition occurs only when cells are treated with mitomycin C during induction and has a limit value of about 50%, independent of mitomycin C concentration. This limit value does not depend on cell heterogeneity since genetically homogeneous subclones, derived from DS19 clone, show levels of mitomycin C inhibition between 16 and 50%. Treatment with mitomycin C at different times after dimethyl sulfoxide addition shows that cell sensitivity to inhibition is not homogeneous during the induction period; it is maximal between 18 and 24 h from the start of induction and is observed with a concentration of mitomycin C as low as 25 nM. The inhibition of the benzidine positive phenotypic expression appears irreversible since this effect is observed on cells even several generations after those which were actually treated.

INTRODUCTION

Murine erythroleukemia cells are virus transformed erythroid cells blocked in a relatively early stage in the pathway of differentiation (1). Erythroid differentiation can be induced in them in vitro by culturing in the presence of one of the several known inducers such as dimethyl sulfoxide (2), hemin (3), hexamethylenepasicametamide (4), or butyric acid (5). Some inhibitors of DNA and RNA synthesis synergistically stimulate erythroid differentiation (6). The induction causes chains of events that eventually lead to synthesis and accumulation of hemoglobin. Increase of globin mRNAs synthesis, production of enzymes of heme biosynthesis, and erythrocyte specific membrane alterations are common effects of the induction, although different transitory metabolic changes might occur depending on the inducer used (7). The use of DMSO as an inducer causes a transient arrest of the cells in G1 before they become definitively committed (8) and it affects the chromatin structure (8, 9). It has already been reported that the intercalating dye propidium iodide differentially binds to the chromatin of MEL cells cultivated with and without DMSO (8). MEL cells induced to differentiate with DMSO are a convenient model system to investigate the step(s) involved in alterations of the chromatin states during the differentiation process. MitC has been chosen in this study because it interacts with DNA, as a single or double strand reagent (10), with preferential target sequences, leading to alterations in the DNA structure (11). MitC causes accumulation of hemoglobin (6) in the T3-C12 clone of murine leukemia cells at concentrations much higher than those compatible with survival of the MEL cell clone we use in this study. We show here that MitC inhibits induction of the benzidine positive phenotype of MEL cells at concentrations that do not affect cell viability and proliferation. The inhibition value is about 30–50% in the original DS19 clone and ranges between 16 and 50% in all its subclones tested. This effect appears to be irreversible. A well defined time period during the induction process has been identified in which the cells show maximal sensitivity to MitC. The high efficiency of the inhibition and the noncorrelation with the concentration or with the duration of cell exposure to the drug are discussed as evidence that MitC, under the conditions used, interferes with MEL cell commitment.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Murine erythroleukemia cells were originated in Dr. Friend's laboratory (1). Clone DS19 was grown in modified Dulbecco's minimal essential medium (Gibco Laboratories, Paisley, Scotland) supplemented with 7.5% fetal bovine serum (Flow Laboratories, Irvine, Scotland), penicillin (550 units/ml) and streptomycin (75 units/ml) at 37°C in 10% CO₂ humidified atmosphere. Cell growth was monitored daily by counting cells in a Burker chamber. Cell viability was measured as the ability to exclude trypan blue (Burr, BDH Chemicals, Poole, England). Cloning of DS19 was performed in 0.4% agar (Difco Bacto-agar). Single colonies, picked up with Pasteur pipets, were enlarged over a period of 30 days and then tested for the expression of the benzidine positive phenotype in the presence or absence of MitC. Experiments were performed also on subclones of individual clones. Inductions were performed by subculturing 5 x 10⁴ cells/ml in fresh medium containing either 280 mM DMSO (Riedel, Hannover, Germany) or 0.1 mM hemin (Sigma Chemical Co., St. Louis, MO). Solutions of MitC (Sigma) in water were filter sterilized and stored at −20°C. Cultures containing MitC were manipulated in dim light to avoid the drug decay. At the end of MitC treatment, cells were washed once by centrifugation and then were resuspended in prewarmed fresh culture medium.

Determination of Benzidine Positive Cells. Percentage values of benzidine positive cells were determined with the following procedure. An aliquot of culture suspension in the culture medium was treated with an equal volume of 0.2% benzidine HCl (Sigma) in 3% acetic acid; then 10% (v/v) of 40 volumes H₂O₂ was added. When hemin was the inducer, 0.4% of 40 volumes H₂O₂ was added. When hemin was the inducer, cells were washed twice in 0.9% NaCl solution before adding the benzidine stain. The percentage of benzidine positive cells was determined from the actual number of cells scored which exceeded 200/ determination. Each value is the average of at least duplicate determinations on duplicate samples. Spontaneous benzidine positive cell induction, in the presence or absence of MitC, was lower than 2%.

Statistical Test. Student's t distribution for paired values has been applied for the statistical analysis of the data.

RESULTS

MitC interferes with the induction of the benzidine positive phenotype of MEL cells only if present during DMSO treatment. There are no statistically significant differences in the percentage of induced benzidine positive phenotype between control and cells treated for 24 h with 25 µM MitC before DMSO induction was initiated (Table 1A). Increase of MitC concentration decreases cell viability and proliferation and eventually causes cell death but has no effect on benzidine positive phenotype expression of the surviving cells (data not shown). Concentrations of MitC, which have no effect if added...
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Table 1 Effect of MitC on the expression of the DMSO induced benzidine positive phenotype in MEL cells

A. Cells were cultivated in the presence of 25 pm MitC for 24 h and then resuspended in fresh medium without MitC and induced with DMSO.

B. Cells were treated with 25 pm MitC during the initial 24 h from the start of induction with DMSO and then were resuspended in fresh medium containing only DMSO. Values are percentage of cells expressing the benzidine positive phenotype between days 4 and 8 following DMSO induction and are the average of at least 4 independent determinations. In A, the differences between control and MitC treated cells are not statistically significant: (P > 0.01); in B, the differences are statistically significant: (P < 0.01).

<table>
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Fig. 1. Effect of MitC on percentage of MEL cell benzidine positive (B+) phenotype expression induced by hemin. Control: cells grown in the presence of 100 μM hemin as inducer (○); test: cells grown as in control plus 25 pm MitC (●). Percentage of benzidine positive cells is determined from days 3 to 6 of treatment. Differences in percentage of benzidine positive cells between control and test are not statistically significant (P > 0.5). Top, growth curves.

Fig. 2. Effect of MitC on percentage of MEL cell benzidine positive (B+) phenotype expression induced by DMSO. Control: cells grown in the presence of 2% DMSO. MitC concentrations of 250 or 2500 pm MitC. Cells grown in the presence of 2% DMSO. MitC concentrations of 250 or 2500 pm in the medium for the initial 4 days of culture. Percentage of benzidine positive cells is determined from days 5 to 8 after DMSO treatment. Differences between control and treated cells are statistically significant (P < 0.01). Top, growth curves.

Fig. 3. Effect of MitC on percentage of MEL cell benzidine positive (B+) phenotype expression. Cells were grown in 2% DMSO. MitC (25 pm) added for the following different 24-h periods during the first 4 days after start of DMSO treatment. Control: ○, 0–24 h; ▴, 24–48 h; △, 48–72 h; ■, 72–96 h. Differences in percentage of benzidine positive cells (B+) between all control and treated cultures are statistically significant (P < 0.01). Top, growth curves.

mitC is an inhibitor of the expression of the benzidine positive phenotype. Treatments during 0–24- and 24–48-h periods cause maximal and almost identical inhibitions while 48–72- and 72–96-h periods are much less sensitive to inhibition of the benzidine positive phenotype induction (Fig. 3). Differential sensitivity of MEL cells to MitC inhibition is present also during the initial 48 h. This is shown by experiments in which MEL cells are exposed to MitC for only 6-h periods during the initial 30 h of DMSO induction. The results of these experiments are reported
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in Fig. 4. It is evident that MEL cells are not equally responsive to MitC in the initial 48-h period of DMSO induction since there are clear differences in the inhibition during the different 6-h periods. Statistical analysis of the data, using matched pairs Student's t distribution, shows that the benzidine positive phenotype is inhibited by MitC during all 6-h periods tested and the period between 18 and 24 h is by far the most effective.

MitC, at the concentrations used in the above experiments (2500–25 pm), influenced neither the cell viability, as indicated by trypan blue exclusion test, nor the cell growth rate as evidenced by the growth curves shown in the figure insets. No significant difference is observed in growth rate between control cells and those treated with MitC for 6-h periods, as determined every 24 h since drug treatment (Fig. 4). Experiments were performed with decreasing MitC concentrations in order to study the correlations between the drug dose and its effect (Fig. 5). The MitC effect is the same between 250 pm and 25 fm. At the lowest concentration value causing inhibition, it is in a ratio of a few hundred molecules per MEL cell. At 0.25 fm, the lowest concentration value tested, MitC did not affect viability, growth rate, and percentage of benzidine positive cells in DMSO induced cells. The effect of MitC was tested also on individual subclones derived from one of the clones of DS19 in our stock, by exposing the cells to 25 pm MitC for 6 h, between 18 and 24 h from the beginning of induction. We found that all clones are inhibited by MitC although to different extents (Table 2). The average inhibition value is about 30% and in all clones this value never reaches 100%.

DISCUSSION

The experiments on MEL cells reported here show that very low concentrations of MitC, between pm and fm, having no influence on cell proliferation and viability, cause a decrease in the induction of erythroid differentiation by DMSO. The decrease in the percentage of induction is observed in cells which are several generations later than that actually treated with MitC (see Fig. 3). This demonstrates that the MitC effect is permanent and not due to a lag in the expression of the benzidine positive phenotype. Other substances were reported to inhibit DMSO induced MEL cell differentiation but, in all those cases, the effects were reversible upon removal of the substance (12, 13). The irreversibility of MitC effect shown here may be due to modifications of DNA. In fact, MitC is known to interact in vitro with both single and double stranded DNA, showing preferential binding to specific short sequences (11). It causes in vivo the formation of single strand exchanges in chromatids, when used at m–mm concentrations (14). If the inhibitory effect is indeed due to DNA modifications, then the finding that low doses (hundreds of molecules per cell) of MitC are effective in inhibiting benzidine positive phenotype expression suggests that the targets for MitC are not randomly distributed on DNA (Fig. 5). Consequently, the MitC specificity is dependent on the chromatin structure rather than on DNA sequences (11, 15). The existence of preferential binding regions on DNA of active chromatin has been observed in in vitro studies of human lung cells treated with a nonspecific reagent such as benzopyrene (16). In our experiments, preferential interactions with chromatin altered in conformations are suggested by the fact that the benzidine positive phenotype expression is inhibited only in cells treated with MitC during DMSO induction. In fact no inhibition is seen when the cells are exposed to MitC for 24-h period prior to DMSO (Table 1). In line with this hypothesis, no effect on benzidine positive phe-

![Image](https://example.com/image1)

**Fig. 4.** Effect of MitC on percentage of MEL cell benzidine positive (B+) phenotype expression when present in the culture for 6-h periods. Cells grown in 2% DMSO. MitC (25 pm) added for the following different 6-h periods from start of DMSO treatment. &, control; O, 0–6 h; □, 6–12 h; A, 12–18 h; Δ, 18–24 h; □, 24–30 h. Differences in percentage of benzidine positive cells (B+) between all control and treated cultures are statistically significant (P < 0.001). Inset, growth curves.

![Image](https://example.com/image2)

**Fig. 5.** Effect of different concentrations of MitC on percentage of MEL cell benzidine positive (B+) phenotype expression. Cells were grown in the presence of 2% DMSO; MitC was added between 18 and 24 h after initiation of culture at the following concentrations: Δ, 25 pm; ◇, 2.5 pm; A, 0.25 pm; □, 0.025 pm; &, 0.25 fm and control. Differences in percentage of benzidine positive cells are statistically significant (P < 0.001), except for the 0.25 fm concentration that gives values indistinguishable from those of the control. Growth rate and viability are identical for all cultures.

**Table 2 MitC inhibition of the expression of the benzidine positive phenotype in individual subclones derived from a clone of DS19**

Clones were tested within 30 days from their isolation. Cells were treated with 25 pm MitC between 18 and 24 h following DMSO induction. Other conditions were as in Table 1. Values are averages of two independent determinations. A, controls; B, treated cells. AIC%, average percentage of benzidine positive cell inhibition for a specific clone in the whole period. AID%, average percentage of benzidine positive cell inhibition for all tested clones in each day. Differences between controls and MitC treated cells are statistically significant (P < 0.001).

<table>
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AID% 36  26  28  30
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... of DS19 clones were sensitive to MitC inhibition with an... to a heterogeneity in the cell population of the DS19 clone as clearly... cell commitment of Friend erythroleukemia cells to erythroid dif... ...summary all the results strongly suggest that the effect of MitC... ...with that expected in the case of MitC interaction with chromatin at specific sites exposed stochastically on the chromosomes during the process of DMSO induced commitment. In summary all the results strongly suggest that the effect of MitC is due to interference with cell commitment.

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