Induction of Differentiation of HL-60 Cells by Dimethyl Sulfoxide: Evidence for a Stochastic Model Not Linked to the Cell Division Cycle

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ABSTRACT

Induction of differentiation of the human promyelocytic leukemia cell line HL-60 by dimethyl sulfoxide (Me$_2$SO) was analyzed to determine the relationship between exposure time of the inducer and cell cycle. A minimum incubation time of 12 hr with Me$_2$SO was required in order to induce differentiation in a small but significant proportion of cells. These expressed differentiation markers (morphology, phagocytosis, and nitroblue tetrazolium reduction) up to 12 hr after Me$_2$SO was removed from the medium. For periods beyond 12 hr and as long as 120 hr of contact of HL-60 cells with the inducing agent, a linear rise in the percentage of differentiated cells was observed. The sensitivity to Me$_2$SO of HL-60 cells synchronized by double thymidine block was examined and found to be similar to that of nonsynchronized cells. The effect of Me$_2$SO was not altered when incubated with cells at different phases of the cell cycle. Finally, even nonproliferating cells were sensitive to the inducing effect of Me$_2$SO. The data are consistent with a stochastic model of induction to differentiation without having any linkage to the cell cycle.

INTRODUCTION

The HL-60 cell line, derived from a human promyelocytic leukemia (4), is a unique human leukemic cell line capable of terminal differentiation in the presence of several inducing agents (3, 5). Me$_2$SO is one of the most studied differentiation inducers. The morphological, biochemical, and immunological changes that it induces in HL-60 cells have been described recently (2, 7, 11). The leukemic promyelocytes of HL-60, when cultured in the presence of Me$_2$SO, differentiate into mature cells of the myeloid lineage, displaying functional characteristics associated with normal peripheral blood granulocytes such as chemotaxis, phagocytosis, and nitroblue tetrazolium reduction. In this study, we have studied: (a) how long an exposure to Me$_2$SO is necessary to induce differentiation of HL-60 cells; (b) whether the differentiation of induced cells is an irreversible process that continues in the absence of the inducer; and (c) whether the commitment to differentiation by Me$_2$SO is related to the cell cycle.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. HL-60 cells were passaged twice weekly in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum (Reheis, Phoenix, Ariz.) in 7% CO$_2$ atmosphere at 37°C. The experiments were performed on cells between passages 30 and 48.

Induction of differentiation was obtained by seeding the cells at a concentration of 5 x 10$^5$/ml in the presence of Me$_2$SO (Microbiological Associates, Rockville, Md.) at a final concentration of 1.2% (v/v) for various periods of time. After exposure, the cells were resuspended in Me$_2$SO-free medium.

Cell counts were performed in a Burker chamber, and viability was assessed by trypan blue dye exclusion. For morphological assessment of the cells, Cytospin slide preparations of aliquots of cell suspensions were prepared using a Cytospin centrifuge and stained with Wright-Giemsa.

NBT Reduction Test. The ability of cells to generate superoxide anion was demonstrated by the reduction of the soluble NBT to blue-black insoluble formazan (25). One ml of cell suspension was incubated for 20 min at 37°C with an equal volume of 0.2% NBT (Sigma Chemical Co., St. Louis, Mo.) dissolved in phosphate-buffered saline (pH 7.2; 0.15 M without Ca$^{++}$, Mg$^{++}$), in the presence of 200 ng of 12-O-tetradecanoylphorbol-13-acetate, obtained from the NIH carcinogen repository (6). Scores of NBT-positive cells were performed on at least 500 cells.

Phagocytosis of Activated Yeast. Yeast was activated by incubation with fresh human serum at a concentration of 2 x 10$^9$ particles/ml, for 20 min at 37°C. Yeast particles were then resuspended in phosphate-buffered saline at a concentration of 10$^9$/ml. Cells were incubated in RPMI 1640 at a concentration of 10$^5$/ml, with activated yeast particles (10$^9$/ml) for 30 min at 37°C. The percentage of cells which have phagocytosed was determined in a hemocytometer by counting those containing one or more yeast particles in their cytoplasm on at least 500 cells (14).

Synchronization of Cells. HL-60 cells were synchronized with respect to the cell cycle by a modification of the method described by Puck (21). Briefly, cells were incubated for 24 hr in medium containing 2 mw thymidine and then washed and resuspended in thymidine-free media. 

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RESULTS

Cell Growth and Me2SO-induced Differentiation in Nonsynchronized HL-60 Cells. The length of time of contact with Me2SO necessary to induce differentiation was determined by culturing HL-60 cells in the presence of Me2SO for 6, 12, 18, 24, 30, 44, 72, 96, and 120 hr.

All cultures were initiated at a cell density of 5 x 10⁶ cells/ml. After 2 days, a density of 1.1 to 1.3 x 10⁶ cells/ml was achieved in all cultures. By the third day, the growth rate varied depending on the length of exposure to Me2SO. Cells maintained with Me2SO for 6 hr achieved a density of 2.5 x 10⁶/ml at Day 5, while there was a progressive decrease in growth at longer exposure times to Me2SO (Chart 1). Viability was over 90% at days 1 and 2 and between 80 and 90% from Day 3 to Day 5.

The pattern of induction of HL-60 cells by Me2SO was followed for 6 days, with Day 0 as the first day of exposure. As shown in Chart 2, exposure for at least 12 hr was needed to produce minimal increase in the proportion of NBT-positive cells, while an increasing proportion of mature cells was then observed up to the 120th hr.

Cells exposed to Me2SO for 12, 18, 24, and 30 hr showed a maximum peak of percentage of NBT-positive cells (14, 22, 35, and 58%, respectively) after 2 days of culture; cells exposed for 44 hr achieved their peak after 3 days (86%); the highest proportion of NBT-positive cells was observed on Day 4 in cultures exposed for 72 hr (92%) and on Day 5 in cultures exposed for 96 and 120 hr (96 and 99%, respectively). A decrease in the percentage of differentiated cells was observed in all cultures after the maximum differentiation peak. Presumably, this is due to proliferation of nondifferentiating cells.

A small percentage of noncommitted cells (1 to 6%) was even present in cultures exposed to Me2SO for as long as 120 hr as shown in Chart 2. These cells when transformed to the Me2SO-free medium were able to reconstitute the normal population of self-renewing HL-60 and displayed a normal Me2SO sensitivity pattern.

The differentiation pattern observed using the ability to phagocytize activated yeasts as a functional criterion showed a curve similar to that observed with the NBT reduction test. The maximum percentage of cells capable of phagocytizing was reached 15 to 20 hr earlier (Table 1).

Morphologically differentiated cells appeared only after 2 days of culture, and their percentage increased, prolonging the time of exposure to the inducer. A comparison of phagocytosis and NBT reduction activities with the morphology of HL-60 cells treated for 30 hr with Me2SO is shown in Table 1.

Synchronization of Cells. Synchronization of cells with respect to the cell cycle was obtained by a double thymidine block as described in the "Materials and Methods." At the end of the second exposure, the cells were released from the blockade and monitored for DNA synthesis, MI, and cellularity.

![Chart 2. Differentiation pattern of nonsynchronized HL-60 cells detected in the 6 days after Me2SO exposure. Differentiation is expressed as the percentage of NBT-positive cells. Various curves refer to cultures incubated for different periods with the inducer.](chart)

![Chart 1. Cell growth of nonsynchronized HL-60 cells after different exposure periods to Me2SO. ○, 6 hr exposure; ○, 30 hr exposure; ▲, 72 hr exposure; △, 120 hr exposure.](chart)


[3H]Thymidine incorporation reached a peak in about 5.5 hr (Chart 3a), and the M1 peak was reached at about 9 hr (Chart 3b). The second thymidine incorporation peak occurred at about 16 to 18 hr and was broader.

The length of each phase was estimated according to the method of Volpe and Volpe (29). In our experimental conditions, the average duration of S was 5.5 hr, that of G2 was 3 hr, and that of M was 1.5 hr. G1 ranged between 7 and 8 hr, and the total cell cycle time was about 16 to 18 hr; the second DNA peak was too widely spread to permit its precise determination. However, the sharp first peak of S phase and the subsequent M phase clearly demonstrate the high rate of synchrony achieved with the double block. Synchronization is further supported by the doubling of cell density observed shortly after the mitosis peak (Chart 3c).

No changes in the average phase duration were noted in cells that proceeded through synchronized S, G2, M, and G1 phases in the presence of Me2SO, and cell viability remained over 90%.

**Effect of Me2SO on Synchronized HL-60 Cells.**

Synchronized cells were released from the thymidine block and resuspended into fresh medium containing 1.25% Me2SO. Cells were maintained in the presence of the inducer for the same periods of time assayed in nonsynchronized cells, i.e., 6, 12, 18, 24, 30, 44, 72, 96, and 120 hr.

Identical proliferation was observed in all cultures during the first 2 days, and doubling was achieved in 10 to 12 hr. From Day 3, growth appeared to depend on the time of exposure, as observed in nonsynchronized cells. Phagocytosis activity, morphological changes, and NBT reduction ability showed a pattern of appearance similar to that of the nonsynchronized cells.

Thymidine alone induced differentiation in a low percentage of cells (12 to 19% of NBT-positive cells), even in the absence of Me2SO. No change in the amount of differentiated cells was observed when synchronized cells were incubated for 6 hr in the presence of Me2SO, while a significant increase was detected after 12 hr of Me2SO exposure. Prolonging the time of incubation with Me2SO increased the proportion of differentiated cells (Chart 4). For incubation times up to 30 hr, the percentage of NBT-positive cells was slightly higher in synchronized than in nonsynchronized cells, whereas afterwards the differences were undetectable. The linear rise in the percentage of differentiated cells, observed in both synchronized and nonsynchronized cells with increasing periods of Me2SO exposure, is compared in Chart 5.

In order to determine if Me2SO promotes expression of myeloid differentiation by an action related to a specific phase of the cell cycle, synchronized cells were exposed to Me2SO for 12 hr at different stages of the cell cycle: (a) immediately after the release of the block (during S-G2 and M phases); (b) 6 hr later (during G2-M and G1 phases); and (c) 12 hr later (during G1 phase and the second S phase). The same amount of differentiated cells were detected in each culture (Table 2), indicating that the effect of Me2SO was not altered when exerted at different phases of the cell cycle.

The action of Me2SO was then tested on cells blocked by a double thymidine exposure. At the 10th hr of the second thymidine exposure, Me2SO was added and was maintained...
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Differentiation of the 30th and the 72nd hr. Thus, 2 sequential events can be noted between induction by Me₂SO and the appearance of morphological changes, in agreement with the findings of Collins et al. (6). However, synchronized cells showed a response pattern that no more cells were committed to differentiation in 12 hr after the Me₂SO was removed.

**DISCUSSION**

The pattern of differentiation induced in cultured HL-60 cells by Me₂SO was determined using both morphological and functional criteria. A close correlation was found between the percentage of cells induced to differentiate morphologically beyond the promyelocyte stage and the percentage of functionally mature cells as described by Collins et al. (6). However, the development of functional markers preceded the appearance of morphological changes, in agreement with the findings of Newburger et al. (18).

A detectable increase in the percentage of differentiated cells was observed only after 12 hr of continuous incubation in the presence of Me₂SO. Exposure for 12 hr is therefore the minimal requirement to promote terminal myeloid differentiation in a significant fraction of HL-60 cells. However, a latent period was noted between induction by Me₂SO and the appearance of differentiated functions, since cells exposed for 12, 18, and 24 hr and then transferred into Me₂SO-free medium showed morphological and functional differentiation markers between the 30th and the 72nd hr. Thus, 2 sequential events can be distinguished in vitro differentiation. They are transition from an uncommitted to a committed state, which requires the presence of Me₂SO, and then the expression of biochemical functions associated with a differentiated phenotype. This second event probably does not require Me₂SO.

<table>
<thead>
<tr>
<th>Time of exposure (hr)</th>
<th>Cell cycle phases</th>
<th>NBT-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12</td>
<td>S-G1-M</td>
<td>30 ± 4^d</td>
</tr>
<tr>
<td>6-18</td>
<td>G1-M-G1</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>12-24</td>
<td>G1-S</td>
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a Cells synchronized by thymidine block were incubated for 12 hr with Me₂SO immediately after the block release (0 to 12), 6 hr later (6 to 18), and 12 hr later (12 to 24).

b Cell cycle phases which cells exposed to Me₂SO were going through.

c Differentiation expressed as the maximum percentage of NBT-negative cells detected at Day 2 of culture.

d Mean ± S.D. of 3 different experiments.

for 12 hr in the presence of the nucleoside. The number of cells remained unchanged, and the MI was always less than 0.2%. The cells were then washed and transferred into RPMI 1640-fetal calf serum alone. The subsequent differentiation degree was significantly higher after the addition of Me₂SO to the cultures (6, 55, and 29% NBT-positive cells at the 24th, 48th, and 72nd hr, respectively). This result indicates that a high induction to differentiation can be obtained in nonproliferating HL-60 cells treated for short periods with Me₂SO.

In order to exclude that the commitment event occurs after the thymidine block is released, by residual Me₂SO that might remain in the cells, some cultures were maintained in the presence of thymidine for additional 12 hr, after Me₂SO exposure and then transferred into RPMI 1640-fetal calf serum alone. The time of appearance of differentiation markers was not delayed by the prolonged thymidine incubation, as one would expect if the action of Me₂SO were exerted after the release from the block. In fact, the percentages of NBT-positive cells after the addition of Me₂SO to the cultures were 10, 58, and 30 at the 24th, 48th, and 72nd hr, respectively, indicating that no more cells were committed to differentiation in 12 hr after the Me₂SO was removed.

Although a minimal exposure time of 12 hr was required for significant induction of differentiation, the proportion of committed cells was directly correlated to the length of exposure to Me₂SO, since an increasing number of cells was induced with increasing periods of incubation with the inducer. This can be explained by either a stochastic model for the commitment process or a genetic heterogeneity of the cell population in its sensitivity to Me₂SO. Genetic heterogeneity seems unlikely to explain the pattern of differentiation that we detected in Me₂SO-treated HL-60 cells. We observed, in fact, that a small amount of uninduced cells was still present in cultures exposed to Me₂SO for as long as 120 hr. These cells were able, if transferred to Me₂SO-free medium, to reconstitute a population of self-renewing HL-60 cells, which, when rechallenged with Me₂SO, did not show a diminished sensitivity to the inducer, as would be expected if genetic heterogeneity were the explanation.

We would suggest, then, that a stochastic model underlies the behavior of cultured HL-60 cells. According to this model, commitment events occur randomly in the population with a particular probability. This probability reflects the likelihood that a cell will undergo differentiation under given culture conditions. A stochastic model has been suggested for the commitment to differentiation of the pluripotent hematopoietic stem cell (28). It has also been proposed for Me₂SO-induced differentiation of Friend erythroleukemia cells (9, 19, 22). In addition, a stochastic model is consistent with the initiation of eukaryotic cell replication process (26).

HL-60 cell differentiation could be explained by sensitivity of the cells to the inducer only during a particular phase of the cell division cycle. For instance, cultured cells could be induced to differentiate only when they enter the active proliferating state, and the decision to enter this state might be stochastically controlled. To investigate whether this mechanism is true for HL-60 cells, cells synchronized with a double thymidine block were induced with Me₂SO. In synchronized cells, the percentage of differentiated cells for incubation times with Me₂SO up to 30 hr was slightly higher than nonsynchronized cells. However, synchronized cells showed a response pattern to Me₂SO similar to that of nonsynchronized cells, in that a direct relationship was observed between the percentage of differentiated cells and the length of exposure. If the action of Me₂SO were related to a specific phase of the cell cycle, one would expect a sharp increase in the amount of differentiated cells each time the cells go through the sensitive phase in the presence of the inducer. This was not observed in our experiments. Cells going synchronously through the cycle showed a linear rise in the percentage of induction with increasing periods of exposure. In addition, identical patterns of induction were obtained in cultures exposed to Me₂SO for 12 hr during 3 different periods of the cycle. Both results strongly support the idea that the inducing effect of Me₂SO is not linked to the cell cycle. This hypothesis is further supported by the demonstration that even nonproliferating cells blocked at the beginning of the S phase of the cell cycle can be induced to differentiate by Me₂SO. The higher rate of differentiation that we detected in cultures treated with thymidine and Me₂SO can
be attributed to a cumulative effect, since slight but significant differentiation was observed with thymidine alone.

Our results are consistent with the finding reported by Rovera et al. (23) that transition through the S phase is not necessary for differentiation of 12-O-tetradecanoylphorbol-13-acetate-treated HL-60 cells into macrophage-like cells. The lack of requirement of DNA synthesis for induction of myeloid differentiation of HL-60 cells is supported by several reports using MEL system (12, 13), but not by others (8, 10, 15, 17). While the reasons for these differences in the MEL system are not clear, our experimental approaches using HL-60 cells had no adverse effect on cell viability, cell cycle time, cytokinesis, or the ability of the cells to commit to differentiation.

Therefore, a stochastic model not linked to the cell cycle is proposed as the basis of induction of differentiation of HL-60 cells by Me2SO. Commitment to differentiation occurs in the absence of DNA synthesis. Its molecular basis remains unknown. In Friend erythroleukemia cells, interactions of inducing agents with cell membrane (1, 16), as well as with DNA (24, 27), have been reported. Since Me2SO and other inducing agents can cause cells in every phase of the cell cycle to differentiate irreversibly to a nonproliferating state, it is possible that this system may be useful in studying antileukemic agents which must act by inhibiting cell proliferation in some manner.

REFERENCES

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