Inhibition by Dexamethasone of Commitment to Erythroid Differentiation in Murine Erythroleukemia Cells

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ABSTRACT

The inhibition of erythroid differentiation of murine erythroleukemia cells by dexamethasone (DEX) has been investigated on a clonal basis. At concentrations which had no detectable effect on cell proliferation, DEX rapidly inhibited the dimethyl sulfoxide (DMSO)-induced commitment of individual murine erythroleukemia cells to the differentiation program. DEX did not prevent heme accumulation in cells already committed to the differentiation process. The rate of globin messenger RNA (mRNA) synthesis was reduced in cells treated with DMSO and DEX compared to cells treated with DMSO alone. The reduction in the rate of globin mRNA synthesis was proportional to the reduction caused by DEX in the rate of commitment. DEX inhibition in the rate of commitment and of globin mRNA synthesis of DMSO-treated cells was reversible. Upon removal of DEX, continued DMSO treatment resulted in a rapid increase in both the rate of globin mRNA synthesis and the rate of commitment. The rate of globin mRNA synthesis after DEX release was also proportional to the rate of commitment. These results suggest that DEX exerts an inhibitory effect on heme and globin synthesis by blocking commitment to terminal erythroid differentiation.

INTRODUCTION

The in vitro differentiation of murine erythroleukemia cells is a useful model system for the study of the control of cellular differentiation (5–8, 11, 14, 16, 18, 21, 23, 25, 30). Recently, it has been reported (20, 27) that corticosteroids are effective in blocking the differentiation of MEL cells. In these studies, corticosteroids were shown to exhibit inhibitory effects on both heme and globin synthesis. Scher et al. (27) demonstrated specific effects on levels of 5-aminolevulinic acid dehydratase and uroporphyrinogen I synthase, 2 enzymes which catalyzed intermediate steps in the synthesis of heme. The activity of each enzyme was reduced by a factor of 10 for MEL cells continuously incubated in DMSO and $10^{-9}$ M hydrocortisone for 118 hr in comparison to MEL cells incubated in the presence of DMSO alone for the same period. Lo et al. (20) demonstrated specific effects of DEX on globin mRNA metabolism. At $10^{-9}$ M DEX, an effect of DEX on globin mRNA entry into polysomes was observed. At $10^{-8}$ M DEX, an effect on the rate of globin mRNA accumulation in the cytoplasm was demonstrated.

The finding that DEX can inhibit DMSO-stimulated differentiation for both heme and globin synthesis suggests that the primary effect of corticosteroids on MEL cells may be to block a central process which controls the complete differentiation program, including heme and globin synthesis. In the present study, we have examined the question of corticosteroid inhibition of MEL cells differentiation from this perspective. Cloning methods which permit determination of whether an individual MEL cell has become committed to inducer-independent differentiation allow a direct measurement of programming of individual MEL cells to the terminal differentiation pathway (11, 16). By analyzing reprogramming of MEL cells to the differentiation pathway on a clonal basis, we show that DEX strongly inhibits the commitment to the erythroid differentiation pathway normally induced by DMSO. The data presented here indicate that the inhibition of the commitment process by DEX is responsible for the multiple inhibitory effects of DEX on heme, globin mRNA, and globin synthesis observed in this system.

MATERIALS AND METHODS

Biological Materials. Cell line 745 was originally obtained from Dr. C. Friend of Mount Sinai School of Medicine, New York, N.Y. 745-PC-4 is a subclone of this line. All liquid cultures were maintained in medium lacking nucleosides (a−) supplemented with 15% v/v fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) in 5% CO2 humidified atmosphere. Fresh medium was used to replenish the culture daily and keep the cell density between $1 \times 10^4$ and $5 \times 10^5$ cells/ml. Under these conditions, untreated cultures were maintained continuously in logarithmic growth. Cell number was determined using an automatic cell counter (Coulter counter Model ZBI, Coulter Electronics, Inc., Hialeah, Fla.).

Chemicals. DMSO was purchased from Mallinckrodt, Inc., St. Louis, Mo. DEX, benzidine dihydrochloride, poly(A), and yeast RNA were purchased from Sigma Chemical Company, St. Louis, Mo. Both poly(A) and yeast RNA were purified by extraction with chloroform:phenol (1:1) mixture as described (1). DEX was dissolved in pure ethanol and added to the culture immediately after cell inoculation. The final ethanol concentration in the cultures did not exceed 0.125% and had no detectable effect on either cell proliferation or differentiation.

Determination of the Proportion of Benzidine-positive Cells. On Day 5 of incubation, the cell cultures were scored for the proportion of hemoglobin-containing cells (benzidine-positive) by staining with benzidine peroxide and a hematoxylin counterstain as described by Gusella et al. (11).

Determination of Hemoglobin Content. To assay hemoglobin content, cells were collected by centrifugation at $350 \times g$ for 5 min, washed twice with phosphate-buffered 0.9% NaCl solution (pH 7.4), and resuspended at a level of $1 \times 10^6$ cells/ml in the same buffer containing 1% Nonidet P-40. Cells were
These observations, which are consistent with those made previously observed (11) for each culture. A detailed analysis of the colony phenotypes was performed as described by McLeod et al. (22) and Gusella et al. (11). In experiments in which the proportion of committed cells was determined, a plating efficiency of greater than 75% was achieved in all cases. Briefly, 100 to 200 cells were plated in 0.1-ml clots in microtiter wells (Linbro Scientific, Inc., Hamden, Conn.) which had been sterilized by UV irradiation. The clots were then incubated at 37° for 20 hr. To this, 2.0 ml of 10% acetic acid was added, and the absorbance was determined at 515 nm within 30 min. Data were calibrated using a beef hemoglobin standard curve.

Plasma Clot Clonal Assay for the Determination of the Proportion of Committed Cells. Plasma clot culture was performed according to the method of Venetianer and Leder (31). In a typical experiment, the reaction mixture (0.5 ml) contained 100 /g yeast RNA, and labeled RNA and cDNA:cellulose as specified in text were collected by centrifugation (1000 x g) for 10 min and resuspended in 20 ml of prewarmed fresh (α~) medium supplemented with 13% fetal calf serum (Grand Island Biological Co.), and 1 mCi of [5-3H]uridine (21 Ci/mmol; New England Nuclear, Boston, Mass.) was added. Two hr later, cells were collected and washed twice with 20 ml of (α~) medium. At the end of labeling period, the cells were collected by centrifugation as above and washed once in (α~) medium supplemented with 10% fetal calf serum (Grand Island Biological Co.). Nuclei were separated from cytoplasm by gentle mixing of the cells in a solution containing 0.25 M sucrose, 0.15 M NaCl, 3 mM MgCl2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0) and 0.5% Triton X-100, and centrifuged at 600 x g for 5 min. The nuclear pellet was washed once more with the same buffer. The combined supernatants of the 2 washes contain the cytoplasm of the cells. Cytoplasmic RNA was extracted 3 to 5 times with phenol:chloroform (1:1) in 0.1 M Tris-HCl (pH 9.0) at room temperature as previously described (1). Globin cDNA cellulose was prepared according to the method of Venetianer and Leder (31).

Preparation of Pulse-labeled RNA and Hybridization with Globin cDNA Cellulose. Control and drug-treated 745-PC-4 MEL cells (approximately 10^6 cells) growing under conditions specified in text were collected by centrifugation (1000 x g) for 10 min and resuspended in 20 ml of prewarmed fresh (α~) medium supplemented with 13% fetal calf serum (Grand Island Biological Co.), and 1 mCi of [5-3H]uridine (21 Ci/mmol; New England Nuclear, Boston, Mass.) was added. Two hr later, cells were collected and washed twice with 20 ml of (α~) medium. At the end of labeling period, the cells were collected by centrifugation as above and washed once in (α~) medium supplemented with 10% fetal calf serum (Grand Island Biological Co.). Nuclei were separated from cytoplasm by gentle mixing of the cells in a solution containing 0.25 M sucrose, 0.15 M NaCl, 3 mM MgCl2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0) and 0.5% Triton X-100, and centrifuged at 600 x g for 5 min. The nuclear pellet was washed once more with the same buffer. The combined supernatants of the 2 washes contain the cytoplasm of the cells. Cytoplasmic RNA was extracted 3 to 5 times with phenol:chloroform (1:1) in 0.1 M Tris-HCl (pH 9.0) at room temperature as previously described (1). Globin cDNA cellulose was prepared according to the method of Venetianer and Leder (31).

Hybridization of [3H]RNA with cDNA:cellulose and elution of labeled RNA was carried out as described by Aviv et al. (2). In a typical experiment, the reaction mixture (0.5 ml) contained 50% deionized formamide, 0.6 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.1% sodium dodecyl sulfate, 1 mM EDTA, 50 µg poly(A), 100 µg yeast RNA, and labeled RNA and cDNA:cellulose as specified. Hybridization was carried out in scintillation tubes shaking in a water bath at 40°. Hybridized [3H]RNA was eluted by 3 successive washes with 1.0 ml of 0.1 N NaOH and neutralized with HCl, and radioactivity was measured in a

Table 1

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Treatment</th>
<th>Colonies with &gt;32 cells (%)</th>
<th>Colonies with &lt;32 cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>None</td>
<td>96.6</td>
<td>0.8</td>
</tr>
<tr>
<td>24</td>
<td>DEX</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>24</td>
<td>DMSO</td>
<td>87.5</td>
<td>3.3</td>
</tr>
<tr>
<td>24</td>
<td>DMSO + DEX</td>
<td>89.0</td>
<td>1.9</td>
</tr>
<tr>
<td>48</td>
<td>None</td>
<td>98.7</td>
<td>0.4</td>
</tr>
<tr>
<td>48</td>
<td>DEX</td>
<td>98.7</td>
<td>0.4</td>
</tr>
<tr>
<td>48</td>
<td>DMSO</td>
<td>39.0</td>
<td>3.4</td>
</tr>
<tr>
<td>48</td>
<td>DMSO + DEX</td>
<td>88.7</td>
<td>2.6</td>
</tr>
<tr>
<td>72</td>
<td>None</td>
<td>98.7</td>
<td>0.8</td>
</tr>
<tr>
<td>72</td>
<td>DEX</td>
<td>98.7</td>
<td>0.3</td>
</tr>
<tr>
<td>72</td>
<td>DMSO</td>
<td>81.1</td>
<td>1.2</td>
</tr>
<tr>
<td>72</td>
<td>DMSO + DEX</td>
<td>80.0</td>
<td>5.1</td>
</tr>
</tbody>
</table>

a: Colonies in which all cells are benzidine reactive.
b: Colonies in which all cells are benzidine negative.
c: Colonies which contained both benzidine-reactive and -negative 745-PC-4 cells were grown and maintained in log-phase (3 x 10^6 to 10^7 cells/ml) conditions in culture with the following additions: none, DEX (1 x 10^-4 M); DMSO (1.5% v/v); and both DMSO and DEX (1.5% v/v and 1 x 10^-4 M). At various times of incubation, cells were removed from the liquid culture and plated in plasma clots in the absence of drugs for 96 hr. Experimental details for plasma culture and scoring the proportion of committed, uncommitted, and mixed colonies are described in "Materials and Methods."

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were observed after 5 days of exposure to DMSO and DEX. Cells were incubated an additional 4 days in the absence of DMSO or DEX. Separate liquid aliquots to the semisolid plasma clot medium, and these cloned aliquots were removed, 200 cells were transferred in 0.1-ml cultures were incubated with DMSO at various concentrations of DEX between 5 x 10^-8 and 5 x 10^-6 M. At various times, the cloning of DMSO- and DEX-treated cells in a semisolid medium can be determined (Table 1). Committed cells give rise to colonies in which all cells are fully hemoglobinized. Because the limitation to proliferative capacity to 5 additional cell divisions accompanies the commitment to terminal erythroid differentiation, such colonies are less than 32 cells in size. The results of this experiment are shown in Chart 2. Within 48 hr, 50% of the cells in a DMSO-treated culture have become committed, and by 70 hr, more than 75% have become committed (Chart 2). Treatment with DEX inhibits the rate of commitment in a dose-dependent manner. Cells treated with DMSO and 5 x 10^-8 M DEX commit to erythroid differentiation at about one-half the rate of cells treated with DMSO alone while cells treated with DMSO and 5 x 10^-6 M DEX never reach a proportion of committed cells higher than 10% after 70 hr of incubation. These results are consistent with the view that a primary effect of DEX on MEL cells is to decrease the rate of commitment to erythroid differentiation and that the block to expression of various differentiated functions is a consequence of the failure of the cells to undergo a process which permits autonomous expression of the differentiation program.

Effects of DEX on Hemoglobin Accumulation. To further test the hypothesis that the primary effect of DEX on DMSO-induced MEL cell differentiation is to reduce the rate at which cells become committed to inducer-independent differentiation an experiment of a different design was performed: the time at which DEX is added in relation to DMSO is varied, and the amount of hemoglobin accumulated is measured (Table 2).
we would expect that DEX treatment should also block the commitment to the differentiation pathway, then treated cultures eventually fail to increase in cell number. If DEX blocks commitment following removal of DEX from the culture and cell plating in the presence of 210 mM DMSO. After 72 hr of incubation with a mixture of DMSO and DEX, removal of DEX resulted in a rapid semisynchronous burst of committed cells, increasing from a proportion of 25 to 75% committed cells within 2 to 4 hr. Removal of DEX at earlier times led to a rapid rate of commitment, but the kinetics exhibited by cultures in which DEX was removed after 24 or 36 hr showed less synchronous commitment than did the kinetics of commitment of a culture switched at 72 hr.

**Removal of the DEX Effect.** To examine more precisely the kinetics with which reversal of inhibition of commitment by DEX occurs, a series of experiments were conducted in which the rate of commitment of a DMSO-treated culture is observed before and after incubation in the presence of $10^{-7}$ M DEX. Results of these experiments are shown in Chart 4. In all cases, cultures treated with DMSO and DEX exhibited a rapid rate of commitment following removal of DEX from the culture and cell number as a function of time are shown in Chart 3. In both experiments, it is clear that while an untreated culture continues to increase in cell number exponentially, a DMSO-treated culture reaches a plateau in cell number after 48 hr of treatment with 210 mM DMSO. Simultaneous treatment with DMSO plus $10^{-7}$ M DEX leads to a continuous increase in cell number over a 144-hr period. The rate of increase in cell number is somewhat less than untreated cells, presumably because with $10^{-7}$ M DEX a small proportion of cells do become committed to erythroid differentiation in each cell generation. If, however, DEX is removed and exposure to DMSO is continued, a subsequent limitation in proliferative capacity is observed. These results indicate that the inhibition of the commitment process is reversible when the steroid is removed.

**Table 2**

<table>
<thead>
<tr>
<th>Agent at 0 to 48 hr</th>
<th>Agent at 48 to 96 hr</th>
<th>Hemoglobin (µg/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0.02</td>
</tr>
<tr>
<td>DEX</td>
<td>DEX</td>
<td>0.03</td>
</tr>
<tr>
<td>DMSO</td>
<td>DMSO</td>
<td>0.85</td>
</tr>
<tr>
<td>DMSO</td>
<td>None</td>
<td>2.31</td>
</tr>
<tr>
<td>DMSO</td>
<td>DEX</td>
<td>2.50</td>
</tr>
<tr>
<td>DMSO + DEX</td>
<td>DMSO + DEX</td>
<td>0.57</td>
</tr>
<tr>
<td>DMSO + DEX</td>
<td>None</td>
<td>0.61</td>
</tr>
<tr>
<td>DMSO + DEX</td>
<td>DEX</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**Effect of DMSO on the proliferation rate of the cells.** Two experiments in which the effect of treatment with DMSO alone and with a mixture of DMSO and DEX on cell number as a function of time are shown in Chart 3. In both experiments, it is clear that while an untreated culture continues to increase in cell number exponentially, a DMSO-treated culture reaches a plateau in cell number after 48 hr of treatment with 210 mM DMSO. Simultaneous treatment with DMSO plus $10^{-7}$ M DEX leads to a continuous increase in cell number over a 144-hr period. The rate of increase in cell number is somewhat less than untreated cells, presumably because with $10^{-7}$ M DEX a small proportion of cells do become committed to erythroid differentiation in each cell generation. If, however, DEX is removed and exposure to DMSO is continued, a subsequent limitation in proliferative capacity is observed. These results indicate that the inhibition of the commitment process is reversible when the steroid is removed.

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**Kinetics of Commitment in Cultures in which DMSO is Added Prior to DEX.** An experimental protocol, which can potentially give some insight into the mechanism of action of
The rate of increase in the proportion of committed cells rises to DMSO, and a final value of 30 times the control (0.3% of input radioactivity) is reached after 36 hr of exposure to DMSO. Treatment of input radioactivity) is reached after 60 hr of exposure to DMSO. OCTOBER 1979 3853

parallel to the rate of globin mRNA production. Treatment of input radioactivity) is reached after 36 hr of exposure to DMSO. For the control culture shown in Chart 5, a lag period of about 12 hr of treatment with DMSO is observed before a significant number of committed cells appear in the culture. Addition of DEX during this period (6 hr) leads to an inhibition in the number of committed cells equivalent to a culture in which DMSO and DEX were added simultaneously. Addition of DEX after 12 hr of DMSO treatment leads to an inhibition of commitment almost as severe, although in this case 5% of the cells become committed after 48 hr of incubation with DMSO. Rapid inhibition in the rate of commitment is observed if DEX is added at 24 or 36 hr of DMSO treatment, although in each case, a 5% rise in the number of committed cells is observed in the 12 hr following the addition of DEX. The major effect in each case, however, is a profound reduction in the rate of commitment shortly after the addition of DEX. These results suggest that DEX acts to block a biochemical event which occurs close in time to the commitment event itself.

Effects of DEX on Globin mRNA Production. If the primary effect of DEX on the differentiation of MEL cells is to block the commitment process, then the effects of DEX on specific biochemical parameters characteristic of the differentiation program should bear a quantitative relationship to DEX effects on commitment. To test this hypothesis, we carried out parallel measurements on the effects of DEX on the rate of globin mRNA production and the rate of commitment in DMSO-treated MEL cells. To measure the rate of globin mRNA production, the cells were pulse labeled with [3H]uridine for 2 hr, and total cytoplasmic RNA was extracted. The labeled RNA was then hybridized to globin cDNA cellulose, and the proportion of the radioactivity bound to the globin cDNA was determined. In cells treated with neither DMSO nor DEX, this value is approximately 0.01% of the input radioactivity (Chart 6). Treatment with DMSO causes a rise in the proportion of the input counts hybridizing. A value of 10 times the rate for untreated cells (0.1% of input radioactivity) is reached after 36 hr of exposure to DMSO, and a final value of 30 times the control (0.3% of input radioactivity) is reached after 60 hr of exposure to DMSO. The rate of increase in the proportion of committed cells rises parallel to the rate of globin mRNA production. Treatment of the cells with $10^{-6}$ M DEX reduces the rate of globin mRNA production and the rate of commitment in the culture dramatically. By 96 hr, values for both of these parameters are approximately 10-fold lower than are the corresponding values for a culture treated with DMSO alone. The inhibition by DEX of commitment of DMSO-treated MEL cells is reversible. If the reduction in the rate of globin RNA synthesis by DEX is due to inhibition of commitment, then DEX inhibition of globin mRNA synthesis should also be reversible. To examine this question, the rates of commitment and globin mRNA synthesis were studied in cultures treated with DMSO and DEX prior to and after the removal of DEX. While continuous presence of DEX in DMSO-treated cultures reduced the rate of commitment and the rate of globin mRNA synthesis at least 10-fold, removal of DEX resulted in a rapid and synchronous increase of both parameters (Chart 7). These data further support the view that inhibition of globin RNA production during DEX treatment is the result of the DEX inhibition of commitment.

DISCUSSION

Glucocorticoids such as DEX appear to affect a wide variety...
of cell types and tissues (15). DEX has been reported to stimulate the proliferation of normal erythroid precursor cells (10). Certain mammary tumors can be shown to increase in growth rate in response to DEX (9). On the other hand, lymphoid leukemia cells are extremely sensitive to DEX. These cells are arrested in G1 prior to cell death when treated with DEX (13). Mouse myeloid leukemia cells are not killed by DEX, however (26). These cells are stimulated by DEX to express certain functions characteristic of differentiated granulocytes and macrophages (26). The effects of DEX on MEL cells contrast with the effects of DEX on these latter cell types. For MEL cells, commitment to differentiation is inhibited, and cell proliferation is favored. While it appears clear that in all cases the interaction between DEX and the cell is likely to be mediated by a steroid receptor protein, the precise mechanism by which DEX treatment alters the proliferation or differentiation of all of these cell types is unknown. Because so many other aspects of the differentiation of MEL cells have been characterized at the molecular level (20), this system may prove useful in the study of the mechanism of action of glucocorticoids in affecting differentiation and proliferation.

Treatment of MEL cells with a variety of chemical agents (3, 7, 12, 17, 19, 21, 24, 28, 29) increases the proportion of MEL cells which commit to terminal differentiation and decreases the proliferative potential of these tumor cells (5, 7, 11, 16, 21). Many of the compounds which are inducers of MEL cells are also effective in causing in vitro differentiation of at least one permanent cell line of human leukemic cells (HL-60) (4). The high degree of correlation between inducers of differentiation of MEL cells and inducers of differentiation of HL-60 suggests that a common molecular mechanism may control the balance between proliferation and differentiation in both cell types. The results presented here suggest that DEX can increase the proliferative potential of MEL cells grown in vitro by reducing the rate at which commitment to differentiation occurs.

The possibility that some human leukemias may exhibit a similar response to glucocorticoids would have significant implications in relation to chemotherapy for these leukemias.

ACKNOWLEDGMENTS

We are indebted to Dr. David Nathan and Dr. Bryan Clarke for reading the paper and providing constructive criticisms. The valuable discussions with Dr. Robert Levenson and David Parker are gratefully acknowledged.

REFERENCES

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