

Erythroid Differentiation in Cultured Friend Leukemia Cells Treated with Metabolic Inhibitors

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SUMMARY

The induction of erythroid differentiation in the T3-C12 clone of Friend leukemia cells by dimethyl sulfoxide is accompanied by reduction in viral RNA-dependent DNA polymerase activity with increased cellular δ -aminolevulinic acid synthetase activity and hemoglobin synthesis. These cells were treated with a variety of compounds to determine whether other drugs are capable of inducing erythroid differentiation. While several hormones, inhibitors of RNA synthesis, organic solvents, inhibitors of DNA polymerase, sulfhydryl inhibitors, and inducers of δ -aminolevulinic acid synthetase administered singly did not stimulate hemoglobin synthesis like dimethyl sulfoxide, inhibitors of DNA and RNA synthesis such as adriamycin, mitomycin C, and hydroxyurea; mithramycin were synergistic in stimulating erythroid differentiation.

INTRODUCTION

Erythroid differentiation can be induced in Friend leukemia virus-infected proerythroblastoid cell lines when incubated with DMSO¹ (7, 10, 11, 15). Differentiation in DMSO-treated cells is accompanied by increases in δ -aminolevulinic acid synthetase activity [the control and limiting enzyme in the heme biosynthetic pathway (9)], heme and globin production, ⁵⁹Fe uptake, and mouse erythrocyte membrane-specific antigen (4, 7, 10, 14, 15). The hemoglobin synthesized *in vitro* is identical to that found in the mouse strain from which the cells were derived (15). Although C-type virus production is elevated in the DMSO-treated cultures (11, 17), these cells exhibit a lower malignant potential than the untreated cultures (7). Erythroid differentiation in DMSO-treated cultures was preceded by a decrease in template-directed RNA-dependent DNA polymerase activity in released Friend leukemia virus (3). We have attempted to mimic the inducing capacity of DMSO with other drugs to elucidate critical steps in the induction of erythroid differentiation. Through the use of various hormones, metabolic inhibitors, and organic solvents, we have investigated which synthetic pathways are altered before hemoglobin production is induced, and we studied further the association of reduced viral DNA polymerase activity with erythroid differentiation.

¹ The abbreviations used are: DMSO, dimethyl sulfoxide; cyclic AMP, cyclic adenosine 3':5'-monophosphate.

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MATERIALS AND METHODS

Cells and Incubation Conditions. The Friend leukemia cell strain T3-C12 (10) was cultured in Roswell Park Memorial Institute medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2 mM glutamine, 10% heat-inactivated fetal calf serum, and penicillin:streptomycin:neomycin (10:10:20 μ g/ml). Stock cells, harvested at a concentration of approximately 10^6 /ml, were split to 0.5 to 1×10^5 /ml when used as untreated control cells or 2×10^5 /ml for drug-treated cultures and the indicated agents were added 3 to 4 hr later. Cell counts were determined in a hemacytometer and the viability was assessed by the trypan blue exclusion method.

δ -Aminolevulinic Acid Synthetase Assays. T3-C12 cells were harvested by low speed centrifugation after 3 days of incubation and were resuspended in 1 mM Tris-HCl (pH 7.4):0.9% NaCl solution at a concentration of 10^7 cells/ml and quick-frozen in dry ice:methanol. Duplicate aliquots each representing 5×10^6 cells were assayed for δ -aminolevulinic acid synthetase activity using 2-keto[5-¹⁴C]glutarate (13.7 mCi/mole; Amersham/Searle, Rockville, Md.) as substrate according to the revised procedure described by Ebert and Pearson (5).

Hemoglobin Assays. On the 5th day of incubation, hemoglobin production in duplicate aliquots of 2×10^6 cells was determined by a modification of the colorimetric benzidine assay procedure of Crosby and Furth (2). The benzidine color from the cells was compared to that obtained from a hemoglobin standard prepared from bovine hemoglobin (2 times recrystallized; Sigma Chemical Co., St. Louis, Mo.).

DNA Polymerase Assays. Particulate DNA polymerase activity in the supernatant fluid from 1 to 2×10^7 cells was determined by the procedure of Scolnick *et al.* (16) after 3 days of incubation. Following removal of cells by centrifugation at $1,000 \times g$ and further centrifugation at $10,000 \times g$, a viral pellet was obtained by centrifuging at $100,000 \times g$ for 50 min. The resultant pellet was disrupted with 0.3% Triton X Buffer A [0.05 M Tris-HCl (pH 7.8):0.05 M KCl:0.001 M manganese acetate] (16) for 10 min at 4°. Following incubation of the enzyme with an incubation mixture containing the template primer poly(rA)-oligo(dT)₁₂₋₁₈ (16) obtained from Collaborative Research, Waltham, Mass., for 30 min, the trichloroacetic acid-precipitable [³H](dTMP)_n was collected on Millipore filters (Millipore Corp., Bedford, Mass.) and counted in the presence of 0.8% 2,5-bis-2-(*tert*-butylbenzoxazolyl)thiophene in toluene. The results were expressed in pmoles [³H](dTMP)_n/10⁷ cells/hr.

Metabolic Studies. T3-C12 cells were split and treated with drugs as described in a previous section. At daily intervals [*methyl*-³H]thymidine (54 Ci/mmole), [³H]-5,6-uridine (40 Ci/mmole), and ³H-reconstituted protein hydrolysate (Schwarz/Mann, Orangeburg, N. Y.) were administered at concentrations of 0.5 μCi/ml, and the flasks were further incubated for 1 hr. Duplicate aliquots of 2 × 10⁶ cells were washed once with 0.1 M Tris (pH 7.4):0.9% NaCl solution. The cells were then treated with 5% trichloroacetic acid, sonically disrupted for 5 to 10 sec, and the precipitates were collected on Millipore filters and counted in 0.8% 2,5-bis-2-(*tert*-butylbenzoxazolyl)thiophene.

Drugs. Hormones, *p*-chloromercuribenzoate, mitomycin C, and puromycin were obtained from the Sigma Chemical Co., St. Louis, Mo., the rifamycin derivatives were obtained from Collaborative Research, Waltham, Mass. Allylisopropylacetamide was a gift from Hoffmann-LaRoche, Inc.,

Kearny, N. J. Actinomycin D was obtained from Calbiochem, San Diego, Calif. Spectranalyzed DMSO was purchased from the Fisher Scientific Co., Philadelphia, Pa. Adriamycin, olivomycin, and chromomycin A₃ were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, Md.

RESULTS

Table 1 lists the different classes of compounds examined to test their potential ability to induce hemoglobin production. Inhibitors of DNA synthesis incubated at concentrations that inhibit cell growth were all effective in stimulating hemoglobin synthesis. Actinomycin D, mithramycin, and cycloheximide exhibited slight stimulation. All other compounds tested except DMSO were inactive.

Table 1
Ability of various agents to induce hemoglobin synthesis in T3-C12 cells
With the exception of the hormones, all agents were incubated with cells up to concentrations that were inhibitory to cell growth. Hemoglobin concentrations were measured at 4 to 5 days.

| Compounds | Concentrations tested [μg/ml or (%)] | Hemoglobin production ^a |
|--|--------------------------------------|------------------------------------|
| DNA inhibitors | | |
| Cytosine arabinoside | 0.005-1 | + |
| Hydroxyurea | 0.01-20 | + |
| Adriamycin | 0.001-0.02 | + |
| Mitomycin C | 0.001-1 | + |
| RNA inhibitors | | |
| Actinomycin D | 0.001-0.1 | ± |
| Mithramycin | 0.1-20 | ± |
| Olivomycin | 0.001-0.1 | - |
| Chromomycin A ₃ | 0.001-0.1 | - |
| Protein inhibitors | | |
| Cycloheximide | 0.01-1 | ± |
| Puromycin | 0.01-10 | - |
| Hormones | | |
| Testosterone acetate | 5-10 | - |
| Insulin | 2.5-5 | - |
| Hydroxycortisone acetate | 10-20 | - |
| Etiocolanolone | 10-40 | - |
| 17 α-Methyltestosterone | 1-10 | - |
| β-Estradiol | 0.001-0.01 | - |
| Progesterone | 10 | - |
| Dexamethasone | 1-10 | - |
| DNA polymerase inhibitors | | |
| 4-N-Dimethylrifamycin | 0.1-1 | - |
| Rifamycin SV | 30-50 | - |
| Rifampin | 50 | - |
| δ-Aminolevulinic acid synthetase inducers | | |
| Barbital | 10-200 | - |
| Allylisopropylacetamide | 100-1000 | - |
| Sulfhydryl inhibitors | | |
| Iodoacetamide | 1-6 | - |
| <i>p</i> -Chloromercuribenzoate | 1-100 | - |
| Miscellaneous agents | | |
| DMSO | (0.5-3) | + |
| Ethylene glycol monomethyl ether | (1.5-2) | - |
| Ethanol | (1.5-2) | - |
| Glycerol | (1-5) | - |
| Cyclic AMP | 1-500 | - |

^a -, barely detectable hemoglobin production, 0 to 0.15 μg/10⁶ cells; ±, slight production, 0.1 to 0.5 μg/10⁶ cells (up to 4 times the control); +, potent production with obvious RBC pellet, 0.5 to 3 μg/10⁶ cells.

Table 2

Effect of selected agents upon δ -aminolevulinic acid synthetase and DNA polymerase activities and hemoglobin synthesis in T3-C12 cells

Cell count, cellular δ -aminolevulinic acid synthetase activity, and supernatant viral DNA polymerase activity were measured on the 3rd day and hemoglobin production was measured on the 5th day. In A, the DNA polymerase activity released from untreated cells was 1.8 pmoles $^{3}\text{H}](\text{TMP})_n/10^7$ cells/hr and, in B, was 1.2 pmoles $^{3}\text{H}](\text{TMP})_n/10^7$ cells/hr.

| Drug | Concentration | % Increase of cell count | DNA polymerase activity/ 10^7 cells (% of control) | δ -Aminolevulinic acid synthetase activity (pmoles/ 10^7 cells/hr) | Hemoglobin concentration ($\mu\text{g}/10^6$ cells) |
|---------------|-----------------------------|--------------------------|--|---|--|
| A. | | | | | |
| DMSO | 2% | 1410 | 100 | 172 | 0.12 |
| Actinomycin D | 5 ng/ml | 830 | 25 | 370 | 1.10 |
| Actinomycin D | 10 ng/ml | 312 | 20 | 137 | 0.48 |
| Cycloheximide | 100 ng/ml | 212 | 24 | 120 | 0.47 |
| Cycloheximide | 100 ng/ml | 230 | 10 | 99 | 0.43 |
| Mitomycin C | 0.5 $\mu\text{g}/\text{ml}$ | 162 | 26 | 345 | 0.46 |
| Mitomycin C | 1.0 $\mu\text{g}/\text{ml}$ | 146 | 31 | 422 | 1.01 |
| Cyclic AMP | 500 $\mu\text{g}/\text{ml}$ | 1210 | 102 | 241 | 0.02 |
| Glycerol | 5% | 216 | 297 | 264 | 0.16 |
| B. | | | | | |
| | | 745 | 100 | 94 | 0.13 |
| Adriamycin | 5 ng/ml | 264 | 72 | 276 | 0.48 |
| Adriamycin | 10 ng/ml | 43 | 24 | 468 | 0.86 |
| Adriamycin | 15 ng/ml | 0 | 38 | 523 | 1.07 |
| Adriamycin | 20 ng/ml | 0 | 30 | 629 | 1.12 |

Several reagents with and without the ability to stimulate hemoglobin production were selected for more intensive study. The results are shown in Table 2. Changes in cell count, DNA polymerase activity, and δ -aminolevulinic acid synthetase activity preceded the onset of hemoglobin synthesis. DMSO inhibited cell growth, caused a decrease in DNA polymerase activity, and stimulated δ -aminolevulinic acid synthetase activity and hemoglobin production. Actinomycin D at low concentrations depressed DNA polymerase and δ -aminolevulinic acid synthetase activities but stimulated only a low level of hemoglobin production. Cycloheximide also inhibited DNA polymerase and δ -aminolevulinic acid synthetase activities and initiated a low level of hemoglobin synthesis. However, higher levels of both drugs completely suppressed hemoglobin synthesis. Mitomycin C (0.5 $\mu\text{g}/\text{ml}$) stimulated a doubling of δ -aminolevulinic acid synthetase activity, a low level of hemoglobin synthesis, and a reduction of DNA polymerase activity to one-quarter that of the control. At a concentration of 1 $\mu\text{g}/\text{ml}$, δ -aminolevulinic acid synthetase activity was further stimulated, and the released DNA polymerase activity was again depressed with a concomitant increase of hemoglobin production to almost 10 times that of the untreated cells. Cyclic AMP, an inducer of δ -aminolevulinic acid synthetase in bone marrow cells (1), showed a slight stimulation of δ -aminolevulinic acid synthetase activity but showed no effect on released DNA polymerase activity or hemoglobin synthesis. Glycerol stimulated a slight increase in δ -aminolevulinic acid synthetase activity and a 3-fold increase in DNA polymerase activity, but did not stimulate hemoglobin synthesis above that of the control. Increasing concentrations of adriamycin were more inhibitory to cell growth and showed an inverse relationship to δ -aminolevulinic acid synthetase activity and

hemoglobin production. DNA polymerase activity was reduced in all adriamycin-treated cultures.

Since Friend *et al.* (6, 8) have observed that DMSO markedly inhibits DNA, RNA, and protein synthesis within 24 hr of its administration to cells, we attempted to duplicate this effect with inhibitors that specifically affect these synthetic pathways. The results of administering combinations of inhibitors upon hemoglobin synthesis in T3-C12 cells are shown in Table 3. DMSO stimulated a 35-fold increase in hemoglobin synthesis and inhibited the growth of the cells. Mithramycin or cycloheximide, in low concentrations administered singly, caused no stimulation of hemoglobin synthesis, while a combination of the drugs showed slight stimulation. Hydroxyurea (0.1 mM) alone inhibited cell growth and was able to stimulate hemoglobin synthesis about 20 times the control level. In combination with mithramycin, the rate of hemoglobin production was about 3 times the level of hydroxyurea alone, while cycloheximide was not synergistic with hydroxyurea. In 3 experiments, slight increases were always observed with mithramycin, cycloheximide, and their combination. Hydroxyurea:mithramycin was consistently the most potent inducer of hemoglobin production, comparing favorably with the response elicited by 2% DMSO. The regimen of hydroxyurea:mithramycin:cycloheximide in a few tests produced a greater increase in hemoglobin production than did hydroxyurea:mithramycin, but it was generally more toxic to the cells.

The effect of DMSO and combinations of hydroxyurea and mithramycin on host cell metabolism are shown in Table 4. The synthetic rate was measured by the incorporation of ^{3}H thymidine, ^{3}H uridine, and ^{3}H protein hydrolysate into insoluble product. The rates should not be consid-

Table 3
Effect of hydroxyurea, mithramycin, cycloheximide, and their combinations upon hemoglobin synthesis in T3-C12 cells
 Hemoglobin production was determined after 5 days of incubation. Similar results were obtained in 2 additional experiments.

| Drug | Concentration [μg/ml or (%)] | % increase of cell count | Hemoglobin concentration (μg/10 ⁶ cells) |
|---|------------------------------|--------------------------|---|
| None | | 2760 | 0.02 |
| DMSO | (2) | 355 | 0.71 |
| Mithramycin | 0.2 | 1560 | 0.04 |
| Cycloheximide | 0.1 | 825 | 0.06 |
| Mithramycin + cycloheximide | 0.2 | | |
| | 0.1 | 71 | 0.25 |
| Hydroxyurea | 8 | 595 | 0.37 |
| Hydroxyurea + mithramycin | 8 | | |
| | 0.2 | 146 | 1.00 |
| Hydroxyurea + cycloheximide | 8 | | |
| | 0.1 | 350 | 0.43 |
| Hydroxyurea + mithramycin + cycloheximide | 8 | | |
| | 0.2 | | |
| | 0.1 | 137 | 0.88 |

Table 4
Time course of the effect of DMSO, hydroxyurea, and mithramycin upon the uptake of tritiated thymidine, uridine, and reconstituted protein hydrolysate into DNA, RNA, and protein of T3-C12 cells
 The results are the average of 2 experiments.

| Drugs | Concentration | Incorporation of tritiated thymidine, uridine, and reconstituted protein hydrolysate into trichloroacetic acid-insoluble form (% of control/2 × 10 ⁶ cells/hr) | | | | | | | | |
|---------------------------|---------------|---|-----|---------|-------|-----|---------|-------|-----|---------|
| | | Day 1 | | | Day 2 | | | Day 3 | | |
| | | DNA | RNA | Protein | DNA | RNA | Protein | DNA | RNA | Protein |
| None | | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| DMSO | 2% | 25 | 35 | 55 | 22 | 34 | 23 | 85 | 61 | 43 |
| Hydroxyurea | 8 μg/ml | 87 | 179 | 154 | 115 | 87 | 73 | 122 | 145 | 101 |
| Hydroxyurea + mithramycin | 8 μg/ml | | | | | | | | | |
| | 0.2 μg/ml | 89 | 89 | 107 | 130 | 65 | 74 | 137 | 87 | 84 |

ered absolute since no attempt was made to measure nucleotide pool sizes. DMSO caused an inhibition of the DNA, RNA, and protein synthetic pathways on the 1st and 2nd days of treatment. By the 3rd day DNA synthesis approached the control level, and rates of RNA and protein synthesis began to recover. Hydroxyurea alone produced only a 13% decrease of DNA synthesis 1 day after drug treatment and modest inhibition of RNA and protein synthesis on the 2nd day. Although hydroxyurea:mithramycin generally induces hemoglobin synthesis to about the same extent as DMSO, the combination treatment did not inhibit any of the synthetic pathways as markedly as DMSO. Elucidation of the specific biological mechanisms affected remains to be determined.

DISCUSSION

We have compared with DMSO the ability of various classes of compounds and metabolic inhibitors to induce erythroid differentiation in T3-C12 cells. Some of the reported changes preceding the onset of hemoglobin synthesis in DMSO-treated cells are a lag in the growth rate (7, 10, 11), decreased DNA, RNA, and protein synthesis (6, 8), increased virus production (10, 11, 17), decreased viral DNA

polymerase (3), and increased cellular δ-aminolevulinic acid synthetase activity (4). Since DMSO is a nonphysiological inducer of hemoglobin synthesis in these virus-shedding proerythroblastoid cells, attempts were made to determine whether these events could be affected selectively by a variety of hormones and metabolic inhibitors. The most potent group of agents was that with a primary effect on DNA synthesis.

Despite an increase in viral 60 to 70 S RNA production in DMSO-treated T3-C12 cells compared to controls, DNA polymerase activity decreased after treatment for 2 days (unpublished observations, P. S. Ebert and D. N. Buell). The decrease in enzyme activity occurred prior to δ-aminolevulinic acid synthetase induction and before hemoglobin synthesis increased. A number of agents, such as rifampin, rifamycin SV, puromycin, and mithramycin, also were able to produce a reduction in the released DNA polymerase activity per cell, but were not effective in stimulating hemoglobin production. Although the reduction of released DNA polymerase activity always preceded hemoglobin synthesis, the event does not appear to be the sole factor controlling the initiation of hemoglobin synthesis.

Tanaka et al. (18) have found recently that many highly polar compounds are active inducers of hemoglobin synthesis in Friend clone 745A. In contrast with our results in

T3-C12 cells, Tanaka *et al.* show that several polar compounds can effectively stimulate hemoglobin synthesis without inhibition of cell growth, although DMSO showed the greatest stimulation at concentrations that inhibited cell growth. These investigators suggested that DMSO and certain other polar compounds act by altering the conformation of DNA or a DNA:protein complex. Similarly, our results suggest that an inhibition or disturbance of the normal synthetic rate of DNA and RNA occurs prior to the onset of erythroid differentiation in these cells.

What parameters are altered before hemoglobin synthesis is initiated? Increasing levels of adriamycin show a progressively decreased growth rate, increasing δ -aminolevulinic acid synthetase activity, and a decrease in released DNA polymerase activity per cell. Mitomycin C showed a similar response. Mitomycin C (19) and adriamycin (12, 20) have been shown to inhibit DNA synthesis and also, to some lesser extent, to inhibit RNA or protein synthesis. DMSO inhibits DNA, RNA, and protein synthesis (6, 8; present results) within 1 day of administration. Inhibition of growth rate is not sufficient to induce hemoglobin synthesis, since Friend cells in stationary phase and those treated with many of the inactive agents listed in Table 1 failed to differentiate. Selective partial inhibition of a single synthetic pathway does not efficiently induce differentiation for low concentrations of hydroxyurea and cytosine arabinoside; actinomycin D, cycloheximide, puromycin, and mithramycin elicit little or no hemoglobin induction. Combinations of specific inhibitors affecting DNA and RNA synthesis and single drugs effective against 2 or more synthetic pathways appear to be potent inducers of hemoglobin synthesis. We conclude that the initiation of hemoglobin synthesis in T3-C12 cells requires a reduced growth rate, a reduction in released DNA polymerase activity per cell, a partial inhibition or disturbance of cellular nucleic acid synthesis, and the coordinated sequential gene activation of enzymes in the heme synthetic pathway.

Rencricca *et al.* (13) have recently demonstrated that hydroxyurea can promote the erythroid differentiation of a small number of hemopoietic precursor cells in the marrow of erythropoietically suppressed mice. The effect was not mediated by erythropoietin since antierythropoietin did not block the erythroid response to hydroxyurea. The above data and the information presented in this paper both suggest that hydroxyurea in some way is derepressing genetic information responsible for the induction of enzymes controlling hemoglobin synthesis. The observation that a "DNA-inhibitor" can induce hemoglobin synthesis both in the hyper-transfused mouse and in the Friend leukemia cell system provides evidence that the leukemic cell model has applicability to studies of normal erythropoiesis.

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