

SEQUENTIAL INDUCTION OF HEME PATHWAY ENZYMES DURING ERYTHROID DIFFERENTIATION OF MOUSE FRIEND LEUKEMIA VIRUS-INFECTED CELLS*

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Friend leukemia virus-infected erythroid precursor cells grow in suspension culture as undifferentiated hemocytoblasts, but undergo differentiation along the erythroid pathway when they are treated with dimethyl sulfoxide (DMSO)¹ (1) or other aprotic solvents (2,3), or butyric acid (4). DMSO-treated leukemia cells show the characteristics of the erythroid cells as judged by the histochemical staining of hemoglobin in the cytoplasm (1), the incorporation of ⁵⁹Fe into heme (1), the fluorometric quantitation of heme (2), the absorption spectra of the cell extract (1), and the characteristic electrophoretic migration pattern of mouse hemoglobin on polyacrylamide gels (5). In addition, DMSO-treated cells form a mouse erythrocyte membrane antigen by the 4th day of culture (6) and become agglutinable with phytohemagglutinin (7). Thus these cells represent a very useful model of erythroid differentiation in tissue culture and permit studies of the process of differentiation in continuously dividing erythroid cells which are otherwise not possible when using cells of normal hematopoietic origin.

Ross et al. (8) on the one hand, have shown that RNA sequences complementary to transcripts of mouse globin messenger RNA (mRNA) can be detected in Friend leukemia cells starting 2 days after treatment with DMSO. On the other hand, the appearance of hemoglobin takes place only 3–4 days after the DMSO treatment. The length of time between the appearance of globin mRNA and of hemoglobin suggests the possibility of a requirement for some other factor(s) before the appearance of synthesis of hemoglobin molecules. Since heme is an obligatory component for the formation of hemoglobin, it seemed of considerable interest to assess the capacity of cells to make heme during the erythroid differentiation of the leukemia cells by assaying activities of certain enzymes of the heme pathway and the concentration of heme itself in such cells.

In this paper, data are reported on the heme concentrations and the activities of certain enzymes of the heme biosynthetic pathway both in undifferentiated cells and in differentiated cells. The highly sensitive assays developed for these enzymes have permitted me to make the novel finding that a sequential induction of enzymes of the heme biosynthetic chain takes place during erythroid differentiation of Friend leukemia cells. Evidence is presented that a sequential induction of these enzymes may be due to sequential activation of genes for these enzymes in the cells.

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¹ Abbreviations used in this paper: ALA, δ -aminolevulinic acid; BrdU, 5-bromo-2'-deoxyuridine; DMSO, dimethyl sulfoxide; URO, uroporphyrinogen-I.

Materials and Methods

Friend Leukemia Cells. A clonal line of Friend leukemia cells (T3-C1-2) was obtained through the courtesy of Doctors Y. Ikawa and P. Leder of the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md. This line hardly differentiates spontaneously, but has been reported to synthesize globin mRNA (8), hemoglobin (8), and erythrocyte membrane antigen (6) after treatment with DMSO.

Cells were grown in 250 ml plastic flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) containing 10 ml of Ham F12 medium supplemented with 10% heat-inactivated fetal bovine serum. Penicillin (100 U/ml) and streptomycin (100 μ g/ml) were also added to the medium. Phenol red was not included in the medium since it interferes with the fluorescence assay of heme. Mycostatin (25 μ g/ml) was found inhibitory to the differentiation process and was excluded from the medium. The cultures were incubated at 37°C under a humidified atmosphere of 95% air-5% CO₂. On the day DMSO was to be added, the cells were transferred to a fresh medium at 5×10^4 cells/ml. DMSO (spectral grade; Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester N. Y.) was then added to a final concentration of 1.5%. The cells were incubated as indicated in each experiment for up to 6 days, and were then centrifuged and assayed in duplicate. 5-bromo-2'-deoxyuridine (BrdU) and thymidine were obtained from Calbiochem, San Diego, Calif. Fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.) which had been previously tested for its capacity to support a good erythroid differentiation was heat inactivated at 56°C for 30 min. The complete growth medium was sterilized by filtration through a 0.45 μ m Millipore filter and stored at 4°C.

Assay for δ -Aminolevulinic Acid (ALA)-Synthetase Activity. The activity of ALA synthetase was determined by a minor modification of the radiochemical assay of Strand et al. (9) using 10^7 cells per assay. The incubation mixture contained 100 mM glycine, 50 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 10 mM EDTA, 1 mM pyridoxal 5'-phosphate, 1 mM dithiothreitol, 10 mM Na malonate, 5 mM Na DL-malate, 5 mM Na arsenate, 2.5 μ g/ml antimycin A, 25 mM ATP, 1 mM coenzyme A, 12.5 mM Na succinate, cells, 2 units of succinyl CoA synthetase (1 unit = 1 μ mol succinohydroxamate/mg protein, 37°C, h), and 5 μ Ci of [1,4-¹⁴C]succinic acid (sp act 24 mCi/mmol) per assay. The final vol of the assay mixture was adjusted to 400 μ l and quickly frozen and thawed in dry-ice acetone three times; the mixture was then incubated for 20 min at 37°C. The reaction was stopped with one volume of 8% TCA. ALA was isolated by using DOWEX column chromatography (Dow Chemical Co., Midland, Mich.) (9).

Assay for ALA-Dehydratase Activity. The activity of ALA dehydratase was assayed spectrophotometrically by the micromethod of Sassa et al. (10) using cells derived from a 1 ml cell suspension. Cells were frozen and thawed three times in dry-ice acetone before the incubation. This procedure was necessary to obtain maximum activity of the enzyme.

Assay for Uroporphyrinogen-I (URO)-Synthetase Activity. This enzyme assay was carried out by the microfluorometric method of Sassa et al. (11) using cells derived from a 1 ml cell suspension per assay. Cells were also frozen and thawed before the incubation as described above.

Fluorometric Assay of Heme. Heme concentration was determined fluorometrically with a minor modification of the assay described by Morrison (12). The method is based on the conversion of the heme moiety of hemoglobin to its fluorescent porphyrin derivative by the removal of heme iron under acidic reducing conditions. Cell suspensions containing 10^8 cells were transferred to a 1 ml disposable glass tube (6 \times 50 mm, Kimble Products Div., Owens-Illinois, Inc., Toledo, Ohio). The tubes were then centrifuged at 2,000 g for 5 min. After removal of the supernates, 500 μ l of 2 M oxalic acid was added to the pellet. The mixture was shaken vigorously and immediately heated for 30 min at 100°C by inserting the lower half of the tube into an aluminum block which had been placed in a heating dry-bath (Thermolyne Corp., Dubuque, Iowa). Small marbles were placed on top of the tube to minimize evaporation. A tissue blank containing cells with the oxalic acid but without heating was run to check for the presence of endogenous porphyrins in the cell. Standards were made by adding 10 μ l of hemin solution prepared in 1% (wt/vol) bovine serum albumin-0.01 N KOH-50% (vol/vol) methanol to 2 M oxalic acid solution, then heating as above.

After cooling, fluorescence was determined in a Hitachi-Perkin Elmer MPF2A fluorescence spectrophotometer (Perkin-Elmer Corp., Hitachi-Perkin Elmer Instruments, Mountain View, Calif.) equipped with an R-446 UR photomultiplier. The tubes were inserted directly into a semimicrocell holder with capacity of accepting four tubes (13). The exciting light was at 400 nm

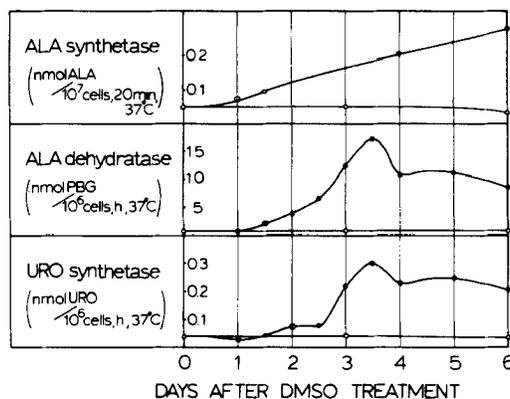


FIG. 1. Changes in the activity of ALA synthetase, ALA dehydratase, and URO synthetase in Friend leukemia cells after DMSO treatment. Cells (T3-C1-2) were grown in the absence (○) or in the presence of 1.5% DMSO (●). There was approximately a 20-fold increase in cell number at the end of the experiment. (⊙), the time when the increase became significant over control. Data are the mean of duplicate ~ triplicate assays.

through blue Zeiss BG-2 and BG-4 filters (Carl Zeiss, Inc., New York), and the fluorescence emission was determined at 662 nm through a red Corning 2-63 filter (Corning Glass Works, Science Products Div., Corning, N. Y.). The response of the spectrophotometer was calibrated against a standard rhodamin B solution (40 ng/ml) in ethylene glycol (14).

The fluorescence generated from heme was strictly linear between 10^{-9} and 10^{-6} M. Under these conditions, $1 \mu\text{M}$ heme gave 15,200 fluorescence units at 662 nm.² The lowest detection of heme in a 0.5 ml mixture by this method was 0.5×10^{-12} mol. Fluorescence yield was linear with cell concentrations up to approximately 2×10^6 cells per assay for both untreated and DMSO-treated cells, but it reached a plateau at cell concentrations greater than 2×10^6 . 1×10^6 cells were used to determine heme concentration in all assays.

Incorporation of ¹⁴C-ALA into Heme. Cells were grown in the presence of $0.5 \mu\text{Ci}$ of ¹⁴C-ALA at a concentration of 2×10^{-5} M and portions of the cell suspension were analyzed for ¹⁴C-heme. Cells were washed three times in Ham F12 medium and 25 μl of carrier hemoglobin (10 g/100 ml) was added. Heme was then extracted with cyclohexanone at pH 2 (15); the extract was dried on a planchette and counted by a gas flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with a counting efficiency of 30%.

Incorporation of ⁵⁹Fe into Heme. Cells were incubated with $0.15 \mu\text{Ci/ml}$ of ⁵⁹Fe (ferrous citrate, sp act 20Ci/g iron) and heme was extracted as described above. ⁵⁹Fe-heme was counted in a Packard Autogamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Results

Induction of ALA-Synthetase, ALA-Dehydratase, and URO-Synthetase activities by DMSO. Activities of ALA synthetase, ALA dehydratase, and URO synthetase were low but detectable in untreated cells. This is in striking contrast to globin mRNA which is reported to be essentially undetectable in untreated cells (8). The levels of these three enzymes in the heme pathway remained constant in untreated cells during the culture period (Fig. 1) and throughout many passages. This confirms that differentiation of these cells toward the erythroid pathway scarcely takes place spontaneously.

² Fluorescence unit is defined as 1/100 division of the full scale recording on a 9 inch chart at the maximal sensitivity. Essentially all the heme was converted to fluorescent porphyrins under these conditions.

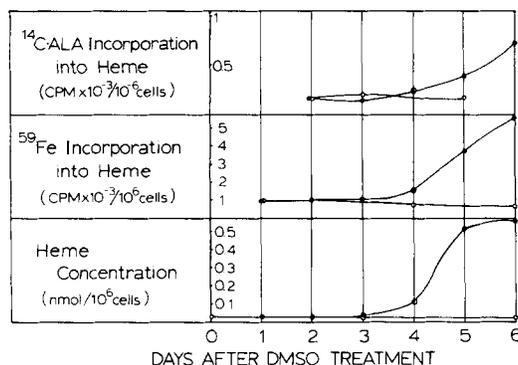


FIG. 2. Changes in the uptake of ALA and iron (Fe) into heme, and heme concentration. Cells (T3-C1-2) were grown in the absence of (O) or in the presence of 1.5% DMSO (●). Data are the mean of duplicate assays.

In striking contrast to the untreated cells, the cells treated with DMSO showed a significant increase in ALA-synthetase activity by the end of the 1st day and ALA-dehydratase activity by 1.5 days (Fig. 1). The activity of URO synthetase showed a significant increase only after the 2nd day, approximately $\frac{1}{2}$ day later than that of ALA dehydratase (Fig. 1). The difference in the time of appearance ALA dehydratase and URO synthetase was consistent and significant since it was always observed when assaying the enzyme activity in the cell suspensions obtained from the same culture flask.

Increase in Heme Concentration by DMSO. The total heme concentration in untreated cells remained approximately constant (0.01 ~ 0.02 nmol/ 10^6 cells) throughout the culture period as well as after many passages over more than a year of study. In cells grown in the presence of DMSO, however, a significant increase of heme concentration became apparent after 4 days of culture (Fig. 2) and a distinct reddish color was readily observed in the pelleted cells by the 6th day. Approximately 0.5 ~ 1.0 nmol heme/ 10^6 cells was found by the 6th day, that is, 50 times more heme per cell than in the untreated cells.

Incorporation of ^{14}C -ALA into Heme. In order to determine when the leukemia cells begin to incorporate ALA into heme, ^{14}C -ALA uptake into heme was investigated. Untreated cells showed a constant level of ^{14}C -ALA incorporation into heme from the 2nd day to the 5th day (Fig. 2). DMSO-treated cells, however, showed an increase in ^{14}C -ALA utilization into heme but only after 4 days of cultures (Fig. 2). Since activities of ALA dehydratase and URO synthetase had already increased by the end of the 2nd day of cultures, ALA incorporation into heme appears to be limited by an enzymatic step(s) which develops later than the appearance of URO-synthetase activity.

^{59}Fe Incorporation into Heme. Untreated cells showed an almost constant ability to incorporate ^{59}Fe into heme throughout the culture period. However, DMSO-treated cells exhibited a significant rise in ^{59}Fe incorporation into heme after the 4th day (Fig. 2). Since this curve of incorporation of ^{59}Fe essentially overlaps with that of ^{14}C -ALA incorporation into heme and meets that of heme concentration as determined fluorometrically, the limiting step for ALA utiliza-

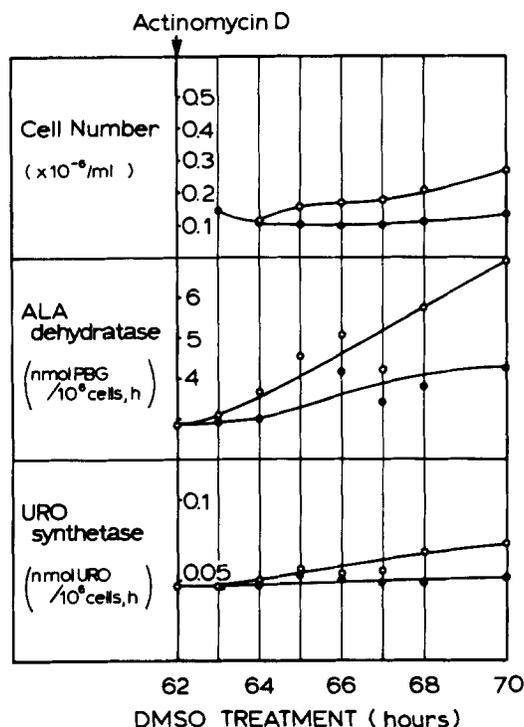


FIG. 3. The effect of actinomycin D on ALA dehydratase and URO synthetase in DMSO-treated Friend leukemia cells. Cells (T3-C1-2) were pretreated with 1.5% DMSO, and actinomycin D ($0.3 \mu\text{g}/\text{ml}$) was added at the 62nd h after the addition of DMSO. Enzyme activities were determined during the following 8 h. (○), 1.5% DMSO. (●), 1.5% DMSO plus $0.3 \mu\text{g}/\text{ml}$ actinomycin D. Data are the mean of duplicate assays.

tion is almost certainly related to the development of ferrochelatase, the terminal step in heme biosynthesis. The increase in ALA incorporation and ^{59}Fe incorporation into heme essentially occurred at the same time and then a significant increase in heme concentration was observed in the cells.

Effect of Actinomycin D on the Activities of ALA Dehydratase and URO Synthetase. In order to investigate whether DMSO-induced changes of heme pathway enzymes are due to increased synthesis of specific mRNA's for these enzymes or due to an increase in translational efficiency of pre-existing mRNA's, cells which had been treated with 1.5% DMSO were incubated with actinomycin D. Since actinomycin D is toxic for cell multiplication, this experiment was carried out between the 62nd and 70th h after the treatment with DMSO. The concentration of actinomycin D used in this experiment ($0.3 \mu\text{g}/\text{ml}$) has been found to inhibit RNA synthesis by 90% in the same clonal line of cells (16).

As shown in Fig. 3, when actinomycin D was added to the culture at the 62nd h after DMSO treatment, it inhibited cell growth almost completely. Cell viability, however, was not appreciably affected as judged by trypan blue exclusion (17). At this time ALA-dehydratase activity increased rapidly in the absence of actinomycin D, whereas in the presence of actinomycin D, the

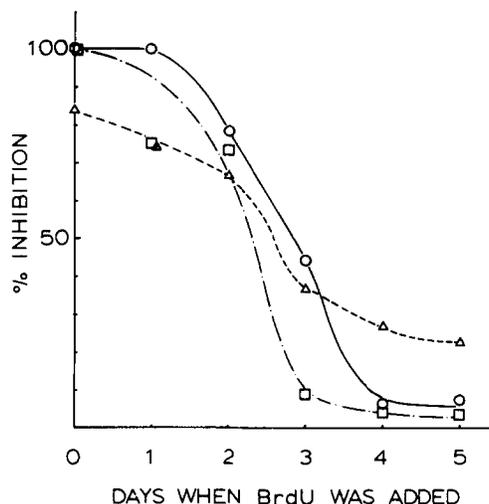


FIG. 4. Inhibition of DMSO-induced changes by BrdU. Cells were grown in the presence of 1.5% DMSO. BrdU (10^{-5} M) was added as indicated in the figure; the enzyme activity and heme concentration were then determined on the 6th day. (○), ALA dehydratase. (△), URO-synthetase. (□), heme. Data are the mean of duplicate assays.

increase in ALA-dehydratase activity was greatly suppressed. Increases in URO-synthetase activity were also prevented by actinomycin D. These results suggest that the increases in the enzymes in DMSO-treated cells are probably due to increased mRNA synthesis for these enzymes in the heme pathway.

Effect of BrdU on the Development of ALA Dehydratase, URO Synthetase, and Heme Concentration. BrdU has been reported to inhibit the formation of globin mRNA (18) and hemoglobin (19) in DMSO-treated Friend leukemia cells. In order to investigate whether BrdU prevents the increase in the activities of ALA dehydratase and URO synthetase, and in heme concentration, BrdU was added to give a final concentration of 10^{-5} M on days 0, 1, 2, 3, 4, and 5 after DMSO was introduced into the cultures; the activities of ALA dehydratase and URO synthetase and heme concentration were determined on the 6th day. When BrdU was added on days 0 and 1, it almost completely blocked the subsequent increases of ALA-dehydratase activity and heme concentration and inhibited the increase of URO-synthetase activity by approximately 80% (Fig. 4). BrdU, however, partially lost its inhibitory effects on the development of these enzymes and heme when it was added on the 2nd day or later after DMSO addition. When the analogue was added at day 5, the inhibition by BrdU of the increases of ALA dehydratase and heme was less than 10% and that of URO synthetase was approximately 25% (Fig. 4). These data suggest, though they do not conclusively establish, that proper transcription is necessary for cell differentiation and the sequential induction of heme pathway enzymes which takes place in these cells.

Effects of Thymidine on the Inhibition by BrdU. When thymidine (7×10^{-5} M) was added in addition to DMSO and BrdU, the inhibitory effects of BrdU on the increases in activities of ALA dehydratase and URO synthetase and the heme concentration were prevented (Fig. 5). The effect of thymidine was great-

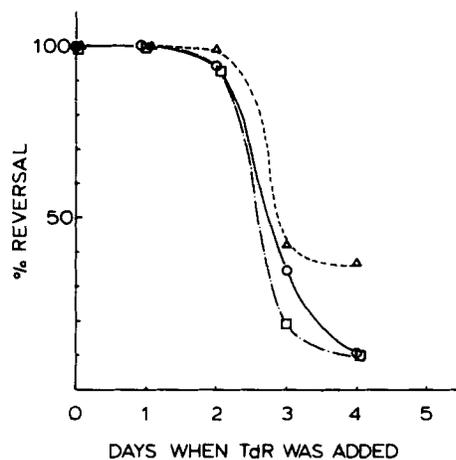


FIG. 5. Reversal of the BrdU inhibition by thymidine (TdR). Cells were grown in the presence of DMSO (1.5%) and BrdU (10^{-5} M). Thymidine (7×10^{-5} M) was then added as indicated in the figure. The enzyme activity and heme concentration were determined on the 6th day. (○), ALA-dehydratase. (Δ), URO-synthetase. (□), heme. Data are the mean of duplicate assays.

est when it was added on days 0, 1, and 2; its ability to reverse inhibition by BrdU was greatly reduced when it was added on day 3 or later. Uridine (7×10^{-5} M) had no effect on the BrdU inhibition.

Discussion

The results of the present study indicate that a sequential induction of enzymes of the heme biosynthetic pathway occurs during erythroid differentiation of Friend leukemia cells according to the biosynthetic order of the enzymes in the pathway. It is clear from this study that the increase in hemoglobin is preceded by a sequential increase of the enzyme activities of the heme biosynthetic pathway. An early increase in ALA-synthetase activity in these cells was found within 24 h after DMSO treatment. Ebert and Ikawa (20) have also reported the early increase in ALA-synthetase activity in these cells by the 28th h after DMSO treatment.

It was found in this study that the activity of ALA dehydratase and URO synthetase in these cells increased within 36 and 48 h after DMSO treatment, respectively. Thus, increases in ALA synthetase and ALA dehydratase in these cells take place as early as the appearance of globin mRNA which has been reported by Ross et al. (8) and precede the increase in hemoglobin concentration by at least 2 days. This fact raises a question whether globin mRNA is not translated into globin peptides or the formation of heme is not completed by the 4th day after DMSO treatment. It has been suggested that undifferentiated Friend leukemia cells contain a translational repressor which might possibly inhibit the translation of globin mRNA (21). However, free globin appears to be made within 2 days of DMSO treatment (W. Scher, personal communication), suggesting that inhibition of translation of globin mRNA is not complete. Moreover, the studies reported in this paper clearly show that ^{59}Fe incorporation into heme increases only on the 4th day after DMSO treatment, suggesting that

ferrochelatase must be limiting in heme formation. In agreement with this idea, ^{14}C -ALA incorporation into heme increased also 4 days after DMSO treatment. Thus, the lag between the appearance of globin mRNA and hemoglobin can be attributed to the late increase in ferrochelatase, the terminal enzyme in the heme biosynthetic pathway.

The increase in activities of the heme pathway enzymes in cells induced to undergo erythroid differentiation apparently depends on de novo RNA synthesis, since actinomycin D blocked these induced changes when added on the 3rd day after DMSO treatment. Moreover, DMSO-induced changes of the heme pathway enzymes appear to require DNA synthesis since they were prevented nearly completely when BrdU was added to the culture and the BrdU inhibition was completely overcome by thymidine but not by uridine. These facts suggest that the BrdU effect on the suppression of induced changes may be mediated through incorporation of the analogue into DNA. It has been also reported by McClintock et al. (22) using a synchronization technique that the Friend leukemia cells require two rounds of DNA synthesis in the presence of DMSO to differentiate.

In studies of the heme pathway of *Staphylococcus aureus*, Tien and White (23) have found by genetic mapping that genes specifying for enzymes of the heme pathway are arranged in the order of their sequence in the biosynthetic pathway. In addition the genes are tightly linked and are co-transducible (23). It is thus tempting to postulate that the genes coding for enzymes of the heme biosynthetic pathway may also be arranged in a sequential fashion in mammalian hemopoietic stem cells and become activated sequentially once DMSO triggers the first gene of the pathway. This hypothesis predicts transcriptional activation of the genes rather than post-transcriptional modification of mRNA's. In support of this idea, as shown in this study as well as in other studies (16), if actinomycin D was also present in the growth medium the heme pathway enzymes did not increase. Also, in cells in which proper transcription was prevented due to BrdU, these enzyme activities of the heme pathway were not increased.

It is not clear how DMSO initiates the first event of the sequential changes. DMSO may alter membrane properties leading to triggering for the "induction." DMSO itself may reach the nucleus of the cell to directly open up the chromosomal genes. Recent evidence in other systems also suggest the possibility that DMSO may directly act on the chromosomes to cause increased transcription (24-27).

From the data presented in this report, it may be concluded that a sequential induction of enzymes of the heme biosynthetic pathway occurs during erythroid cell differentiation in Friend leukemia cells. Our previous work (28) also indicates that similar sequential induction of the heme biosynthetic pathway may occur during erythropoietin-induced erythroid differentiation in the spleen of polycythemia mice and that the induction of ALA synthetase in the spleen by erythropoietin is also prevented by actinomycin D. It has similarly been found during the development of fetal mouse liver that ALA synthetase, ALA dehydratase, and ferrochelatase appear to increase sequentially (29). Since these latter two examples are of erythroid differentiation in a normal hematopoietic tissue,

it may generally be suggested that sequential induction of the heme pathway enzymes occurs when the stem cell, either normal or virally transformed, differentiates into the erythroid pathway.

Summary

The process of erythroid differentiation in mouse Friend leukemia virus transformed cells (T3-C1-2) was examined by following changes in several enzyme activities of the heme biosynthetic pathway and in heme concentration while the cells were undergoing erythroid differentiation after treatment with dimethylsulfoxide. Untreated cells on the one hand, have a limited capacity for spontaneous differentiation. On the other hand, dimethylsulfoxide(DMSO)-treated cells showed an increase in the activities of δ -aminolevulinic acid (ALA) synthetase, ALA dehydratase, uroporphyrinogen-I synthetase, ferrochelatase, and heme concentration by days 1, 1.5, 2, 4, and 4, respectively.

The increase of the heme pathway enzymes and heme concentration followed the order of these enzymes or products as they are arranged in the heme biosynthetic pathway. These changes induced by DMSO were effectively inhibited by treatment with actinomycin D, suggesting that continued RNA synthesis is required for the differentiation process. 5-bromo-2'-deoxyuridine (BrdU) (10^{-5} M) inhibited the DMSO-induced changes of the heme pathway enzymes. BrdU was most effective when it was present during the first 2 days of cell culture. It gradually lost its inhibitory effect when added after the 3rd day or later. The BrdU-mediated inhibition was completely overcome by the addition of thymidine (7×10^{-5} M), but not by uridine (7×10^{-5} M). All these data suggest that a sequential induction of the heme pathway enzyme takes place during erythroid differentiation of Friend leukemia cells, and that the sequential induction of the enzymes may be due to a sequential activation of genes coding for these enzyme activities.

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