THE DEPENDENCE OF ERYTHROID DIFFERENTIATION ON CELL REPLICATION IN DIMETHYL SULFOXIDE-TREATED FRIEND LEUKEMIA-VIRUS-INFECTED CELLS

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Received April 5,1976

Summary — The dimethyl sulfoxide (Me_2SO)-mediated induction of hemoglobin synthesis in Friend leukemia cells (a murine erythroblastoid cell line) is coupled with the number of cell replications occurring in the presence of inducer. Varying concentrations of proflavine increase the generation time of these cells from 24 hours to over 50 hours, and in each case the induction of hemoglobin synthesis follows the completion of two cell doublings. Once the induction is initiated, the rate of hemoglobin accumulation is not affected by proflavine. These data indicate that proflavine does not affect the transcription or translation of globin mRNA and that the delay in induction of hemoglobin synthesis is due to its effect on the rate of cellular replication. In experiments using high concentrations of thymidine to block replication, hemoglobin accumulation is prevented only if the cells are blocked prior to 36 hours after Me_2SO addition. If the cells have completed two generations in the presence of Me_2SO, there is no effect upon their ability to synthesize hemoglobin even though their growth is arrested. Thus, the inhibition of hemoglobin synthesis by proflavine is not merely the result of a toxic effect on newly subcultured cells but is due to its effect on cellular replication. These experiments confirm that, after addition of Me_2SO, Friend leukemia cells require more than one complete cell cycle in order to synthesize hemoglobin.

Friend-virus-induced leukemia cells, when grown in tissue culture, show a limited ability to differentiate along the erythroid cell line (1–3). The incubation of these cells with medium supplemented with 2% (v/v) dimethyl sulfoxide or 1% (v/v) N,N'-dimethylformamide increases the number of differentiating cells in these cultures (2, 4). In addition, it has recently been shown that a variety of highly polar, organic compounds (5), as well as butyric acid (6), will induce these murine erythroblastoid leukemia cells to differentiate along

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§Operated by the Union Carbide Corporation for the U.S. Energy Research and Development Administration.

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the erythroid cell line. In cultures of Me_2SO-treated cells, the following characteristics of differentiating erythroid cells are observed: (i) Globin mRNA (7-9), globin (10, 11), and heme are synthesized; (ii) morphological changes take place that are similar to those seen in maturing erythroid cells (12); (iii) erythrocyte-specific membrane antigens are detected (4); and (iv) as in other differentiating systems, 5-bromo-2'-deoxyuridine is a potent inhibitor of differentiation (13, 14).

Recent studies by McClintock and Papaconstantinou (15) have shown that at least one (but not more than two) round of DNA synthesis and/or mitosis is required for the Me_2SO-mediated induction of erythroid differentiation and hemoglobin synthesis in these cells. In these studies it was shown that, upon extension of the generation time by a variety of methods such as lowering the serum content or treatment with N^6,$O^{2'}$-dibutyryl-adenosine-3':5'-cyclic monophosphate, the synthesis of hemoglobin occurs only after two doublings have occurred. Moreover, in studies with synchronized cultues, it was observed that two mitoses were required for the initiation of hemoglobin synthesis (15). Similar studies by Levy et al. (16) have shown that synchronized cells must be exposed to Me_2SO for at least 24–40 hr in order to initiate hemoglobin synthesis.

We have continued to examine the relationship between cell replication and erythroid differentiation by the use of two agents, proflavine and thymidine. Proflavine has been shown to specifically inhibit the hydrocortisone-mediated induction of glutamine synthetase in embryonic neural retina cells (17, 18) through blocking the formation of functional RNA templates for the de novo synthesis of the enzyme, while blocking no more than 12% of total RNA synthesis. An analogous effect of proflavine on the Me_2SO-stimulated hemoglobin synthesis was sought. Thymidine was used as an effective nontoxic inhibitor of cell replication through its action on thymidine kinase (19).

The results of this study show that proflavine does not inhibit the induction of hemoglobin synthesis by a direct effect on transcription but delays the appearance of hemoglobin by increasing the generation time of the cells. Similarly, inhibition of cellular replication by thymidine has no effect either on the induction of hemoglobin synthesis or on the rate of accumulation of hemoglobin if the cells have traversed the required number of cell cycles prior to the addition of inhibitor. Thus, neither the induction nor the rate of hemoglobin accumulation is affected by proflavine or thymidine, and the use of these inhibitors of replication clearly shows that this cell line requires a specific number of replications in the presence of Me_2SO for the induction of hemoglobin synthesis.

MATERIALS AND METHODS

Cell cultures — The erythroblastoid leukemia cells (clone 745) used in these experiments were cloned from a culture of Friend leukemia-virus-infected cells which was derived from

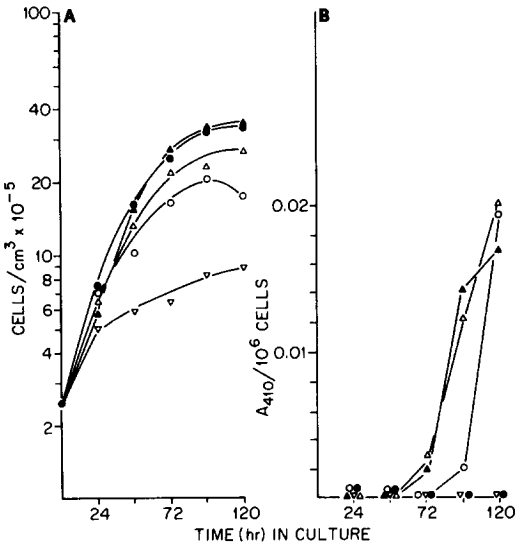

Figure 1. The effect of varying concentrations of proflavine on cell growth and induction of hemoglobin synthesis in Friend leukemia cells. Cultures were seeded with 2.5 X 10^5 cells/cm^3. The concentration of Me_2SO is 2% (v/v). (A) Cell proliferation and (B) hemoglobin accumulation in control cultures (●——●), 2% Me_2SO (▲——▲), 0.2 μM proflavine + 2% Me_2SO (△——△), 0.5 μM proflavine + 2% Me_2SO (O——O), and 1 μM proflavine + 2% Me_2SO (▽——▽).

the spleen of Friend-virus-infected DBA/2J mice (13). The cultures were obtained from the Mammalian Genetics Mutant Cell Repository, Camden, New Jersey, 08103. The cells were grown in Eagle's minimal essential medium (20) with Hanks' balanced salts solution (21) and 15% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). Cell lines were maintained in log phase by subculturing into fresh medium every 3–4 days. All cultures were maintained in plastic flasks (Falcon) at 37°C under humidified atmosphere of 95% air–5% CO_2 in a total volume of 30 ml of culture medium.

Reagents — Proflavine (Mann) was dissolved in Puck's saline A (22) and sterilized by filtration. Dimethyl sulfoxide (Mallinckrodt) was added directly to culture media to a final concentration of 2% (v/v).

Hemoglobin assay and cell counts — Cells in 5-ml aliquots of culture medium were pelletted by centrifugation at 1,000 X g for 10 min, washed twice with saline solution, and frozen at -20°C. To measure hemoglobin, the cell pellet was lysed with 0.5 ml of distilled water, followed by addition of sodium dodecyl sulfate to a final concentration of 2% (w/v). The absorbance of the cleared cell lysates was measured at 410 nm. The A_{410} of lysates of cells cultured with 2% Me_2SO for 48 hr was used as the blank for control of absorbance by nonhemoglobin material. The number of cells per cm^3 was measured by standard hemocytometry.

RESULTS

To study the effect of proflavine on cell growth and on the Me_2SO-mediated induction of hemoglobin synthesis, Friend leukemia cells were incubated in the presence of proflavine ranging in concentration from 0.2–1.0 μM. The data in Figure 1A show that the rate of

cellular replication is reduced by proflavine and the effectiveness of the inhibitor is dose-dependent at concentrations between 0.2 μM and 1.0 μM. The induction of hemoglobin synthesis is delayed by proflavine in a dose-dependent relationship; 0.2 μM proflavine neither alters the time when hemoglobin induction occurs nor the rate of accumulation of hemoglobin after it has been induced (Fig. 1B). This concentration of proflavine does not affect the rate of cellular replication. With a concentration of 0.5 μM the appearance of hemoglobin is delayed by 24 hr and the generation time is increased to over 34 hr. However, the rate of accumulation of hemoglobin is not affected by this concentration of proflavine. Finally, in 1 μM proflavine, there is a complete inhibition of the induction of hemoglobin synthesis, and the generation time was extended so that two generations were not achieved at the end of the experiment. We conclude from these experiments that the effect of proflavine on the induction of hemoglobin is not a direct effect on the synthesis of globin mRNA but a result of its effect on the rate of replication of the cell.

The inhibitory action of proflavine on replication and the induction of hemoglobin synthesis are both reversible. Cells cultured for 48 hr in Me_2SO and 1 μM proflavine, when washed, resuspended in fresh inducing culture medium, and cultured for 120 hr were able to return to a generation time of 22–24 hr and to accumulate hemoglobin at a rate parallel to that of untreated controls.

Other studies have shown that proflavine specifically inhibits the transcription of RNA required for the hydrocortisone induction of glutamine synthetase in embryonic chick retina (17, 18). Our studies indicate that the ability of proflavine to inhibit induction of hemoglobin synthesis is primarily due to inhibition of the cellular replication which is required for the induction, and that transcription of the globin genes is not affected by this inhibitor. To further examine our hypothesis, experiments were done to determine whether proflavine affects hemoglobin synthesis at various times during the induction. A series of cultures was treated with proflavine (1 μM) at 0, 24, 36, 48, 60, and 72 hr after the addition of Me_2SO, i.e., after the initiation of the induction reactions. The growth curves in Figure 2A show that the earlier the administration of proflavine the more reduced the total number of cells at the end of the culture period due to an increase in the generation time. However, the pattern of hemoglobin induction and accumulation (Fig. 2B) indicates that the effect of proflavine is more closely correlated with cellular replication than with transcription. Hemoglobin induction occurs in all the cases shown in Figure 2, regardless of the time of proflavine addition. However, it can be seen that the time of induction is delayed according to the effect of proflavine on the generation time of the cells; when proflavine is added at 0, 24, and 36 hr the generation time is extended and the time of hemoglobin induction is

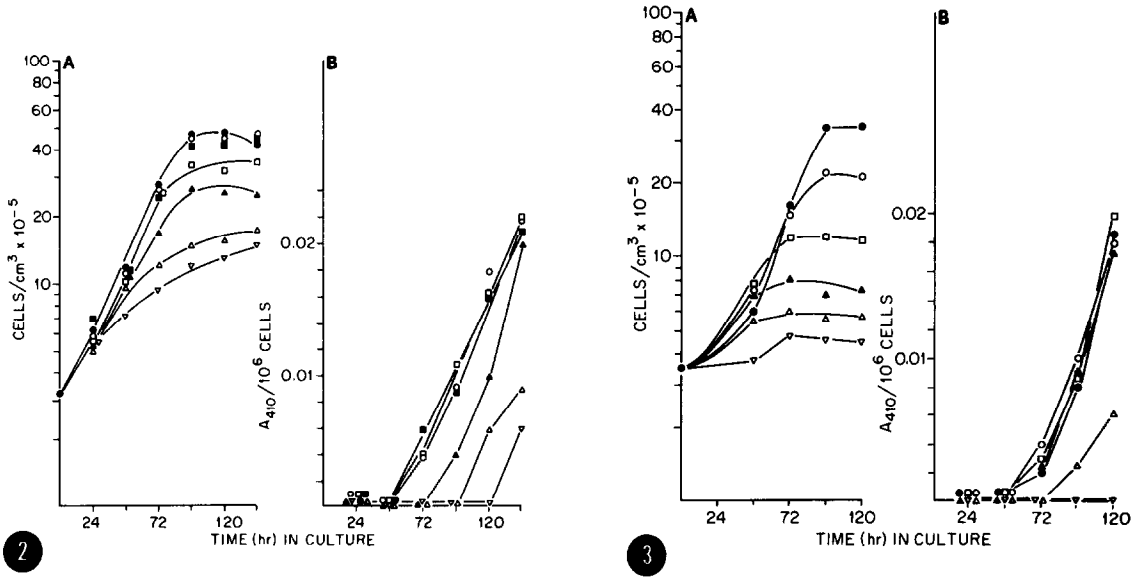

Figure 2. Cell growth, and the induction and accumulation of hemoglobin, in Friend leukemia cells blocked by proflavine at various times during the induction period. Cultures were seeded at a density of 3.2 X 10^5 cells/cm^3 and induced with 2% Me_2SO, which was added to all cultures at zero time. Proflavine (1 μM) was added at the times indicated below. (A) Cell proliferation and (B) hemoglobin accumulation in cells treated with proflavine at 0 hr (▽——▽), 24 hr (△——△), 36 hr (▲——▲), 48 hr (□——□), 60 hr (■——■), and 72 hr (O——O) after the addition of Me_2SO; control (●——●).

Figure 3. Cell growth and accumulation of hemoglobin in Friend leukemia cells blocked by thymidine at various times after the initiation of Me_2SO treatment. Cultures were seeded at a density of 3.5 X 10^5 cells/cm^3. Thymidine (2.5 mM) was added to the cultures at the times indicated below. (A) Cell growth and (B) hemoglobin accumulation in cells treated with 2.5 mM thymidine at 0 hr (▽——▽), 24 hr (△——△), 36 hr (▲——▲), 48 hr (□——□), and 72 hr (O——O) after the addition of Me_2SO; control (●——●).

delayed accordingly. Addition of proflavine at 60 and 72 hr has no effect on the induction of hemoglobin synthesis and essentially no effect on the generation time of the cell, since these cells have already entered stationary phase. In all cases, it can be seen that once induction has occurred, the rate of accumulation of hemoglobin is the same as that seen in control cells. We conclude from these experiments that proflavine does not affect the transcription of globin genes and that the inhibitory effect on induction is associated with the effect on cellular replication.

Through the use of thymidine, it is possible to block cell division with a minimum of the side effects usually inflicted on cells by inhibitors. The ability of thymidine to block cellular replication was used to study the relationship between replication and Me_2SO-mediated induction of hemoglobin synthesis. In this experiment, thymidine (2.5 mM) was added to the cultures at times from 0 to 72 hr after addition of Me_2SO. The data in

Figure 3A show that thymidine effectively blocks the replication of Friend leukemia cells and that the induction of hemoglobin synthesis can be delayed, again depending upon the number of replications the cells completed in the presence of Me_2SO prior to addition of thymidine (Fig. 3B). Cells whose replication was blocked at 0 hr show no hemoglobin synthesis at 120 hr; such cultures when carried up to 218 hr gave no evidence of hemoglobin synthesis. Moreover, by the criterion of exclusion of trypan blue, 85–90% of the cells were viable after 218 hr. The cells in cultures treated with thymidine 24 hr after Me_2SO addition are inducible; however, the induction is delayed by 24 hr. These cells have gone through one cell cycle in the presence of Me_2SO, and the data indicate that this length of exposure to the inducer can elicit a delayed induction. We interpret these data to indicate that only part of the second cell cycle may be needed for the induction of hemoglobin synthesis. Our observation is in agreement with that of Levy et al. (16), who showed that cells of clone 745A must be exposed to Me_2SO for periods longer than 24 hr for the induction of hemoglobin synthesis, and that this must include one critical S phase.

Cultures induced with Me_2SO, and administered thymidine 36 to 72 hr later, all show the same initial appearance of hemoglobin and the same rate of hemoglobin accumulation as is seen in control cells. Thus, in Me_2SO-induced cells cultured for 36 hr before thymidine addition, the cells have been through the critical period of exposure to the inducer and there is no further dependence of induction on continued replication. Thymidine does not stop growth immediately, but within ~24 hr. Thus cells given thymidine at 24 hr actually cease growth at 48 hr, and those treated at 36 hr stop at approximately 60 hr. Slowing cell growth, at any time before 48 hr, delays or prevents hemoglobin synthesis. After 48 hr there is no effect. This gives a critical period of between 24 and 48 hr after addition of Me_2SO.

DISCUSSION

These investigations emphasize recent observations that replication in the presence of inducer is coupled to the Me_2SO-mediated induction of hemoglobin synthesis by Friend leukemia cells (14, 16). The occurrence of a delayed induction in cultures blocked with thymidine after 24 hr in Me_2SO strongly indicates that the second cell cycle need not be completed for the induction of hemoglobin synthesis. On the other hand, the full induction of cultures treated with thymidine at 36 hr indicates that the cells have traversed through the critical phase(s) of the cell cycle and that no further replication is needed for the initiation of globin synthesis. Since the synthesis of hemoglobin in cells continuously incubated in thymidine from 36–120 hr is the same as in control cells, we conclude that thymidine

has no direct effect on either the transcription or translation of globin mRNA, and that the thymidine effect is due to its inhibitory effect on cell replication.

Experiments involving the addition of proflavine to cultures at various times after exposure to Me_2SO suggest that the acridine delays the induction of hemoglobin for a duration that is related to the generation time of the culture. In each case hemoglobin begins to appear in cells after the cell number of the culture has doubled twice. Thereafter the rate of accumulation of hemoglobin is similar in all experimental cases. From these observations we conclude that the proflavine effect on hemoglobin synthesis in Friend leukemia cells is primarily through its effect on cell replication.

Recent studies have indicated that neither DNA synthesis nor mitosis are needed for the butyrate-mediated induction of hemoglobin synthesis by cells of clone T3C12 (23). We have found that butyrate (1.5 mM) also induces hemoglobin synthesis in cells of clone 745. After 24 hr, 23% of the population is benzidine positive (B^+), and the maximum response is reached at 48 hr, when approximately 33% of the population is B^+. This same population of cells responds to 2% Me_2SO after 48 hr, and reaches maximum induction at 5 days, when 82% of the population is B^+. This difference in response to inducers cannot be attributed to toxicity since the growth curves for butyrate and Me_2SO-treated cells are similar. These observations indicate to us that a heterogeneous population can arise from cloned Friend erythroblastoid cells, and that butyrate and Me_2SO may affect different sites in the reactions involving the induction of hemoglobin synthesis.

Proflavine, an intercalating agent, has been shown to specifically inhibit the hydrocortisone-mediated induction of glutamine synthetase synthesis in embryonic chick neural retina (17, 18). It is postulated that, in these cells, proflavine inhibits the formation of functional mRNA templates for the de novo synthesis of the enzyme. The specificity of this inhibition is further emphasized by our observation that proflavine has no effect on either the Me_2SO-mediated induction of globin synthesis or on the rate of accumulation of hemoglobin. It is indeed interesting that this agent, whose inhibitory action is believed to be due to its ability to intercalate with DNA, is not inhibitory to both induction systems, and we interpret this observation as an indication of the existence of significant difference(s) in the factor(s) that is involved in the regulation of transcription of these genes.

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