Regulation of Hemoglobin Synthesis in a Murine Erythroblastic Leukemic Cell: The Requirement for Replication to Induce Hemoglobin Synthesis

(differentiation/Friend leukemia cells)

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ABSTRACT The induction of hemoglobin synthesis by dimethylsulfoxide in cells infected with Friend leukemia virus has been shown to depend on the number of cell divisions after the addition of the inducing agent. In asynchronous cultures the cells have a generation time of 24 hr, and hemoglobin synthesis occurs after 48 hr in the presence of dimethylsulfoxide. Generation times were extended by lowering the serum content of the medium. In 7.5% and 2.5% fetal calf serum, the generation time was increased to 36 hr and 48 hr, respectively, and hemoglobin synthesis began at 72 hr and 96 hr, respectively. In the presence of N\textsubscript{6},O\textsuperscript{\textprime}dibutyryladenosine 3':5'-cyclic monophosphate the generation time was extended to 38 hr, and hemoglobin synthesis began at 72 hr in dimethylsulfoxide.

The number of cell doublings, under conditions where the generation time was 24 hr, was regulated by the size of the initial cell concentration. When a low inoculum (1.5 \times 10^6 cells per cm\textsuperscript{2}) was used, the culture was still in log phase after two doublings and hemoglobin appeared in log phase; a moderate initial inoculum (4.2 \times 10^6 cells per cm\textsuperscript{2}) took the culture into stationary phase after two doublings, and hemoglobin synthesis was induced in stationary-phase cells. Heavy inocula (3.7 \times 10^6 cells per cm\textsuperscript{2}) maintained the cells in stationary phase, and under these conditions no induction of hemoglobin synthesis occurred. We conclude from these studies that the initiation of hemoglobin synthesis required two rounds of mitosis after treatment with dimethylsulfoxide.

In studies with synchronized cultures of cells infected with Friend leukemia virus, two mitoses were required for the initiation of hemoglobin synthesis. We conclude that two rounds of DNA synthesis and/or mitosis are also required for the dimethylsulfoxide-mediated induction of erythroid differentiation and hemoglobin synthesis in these cells.

Friend leukemia cells (FLC), when grown in tissue culture, can be induced to differentiate along erythroid lines (1–3). The induction can be stimulated by the addition of 2% (v/v) dimethyl sulfoxide (Me\textsubscript{2}SO) (2) or 1% (v/v) dimethylformamide (4) to the culture medium. The most striking manifestation of this differentiation is the appearance of mouse hemoglobin in the cytoplasm. In addition, Me\textsubscript{2}SO induces the appearance of erythrocyte-membrane antigen in at least one FLC line (5). Mouse globin messenger RNA has been detected in the cytoplasm of Me\textsubscript{2}SO-treated FLC (6, 7), and a 100-fold increase in virus production has been reported (8, 9).

After about 3 days of exposure to Me\textsubscript{2}SO, the cells have a discernible pink tinge, and after 5 days they are quite red.

RNA sequences complementary to transcripts of mouse globin mRNA can be detected in FLC after 2 days of exposure to Me\textsubscript{2}SO, but little or no hybridization is observed in uninduced cells (6). The hemoglobin produced is reportedly the normal adult form for DBA/2 mice (3, 10) (the strain from which the FLC are derived).

The length of time between addition of Me\textsubscript{2}SO and the appearance of hemoglobin suggested the possibility of a requirement for a precise number of mitoses before the expression of differentiated functions. Since other differentiating systems have been shown to depend on DNA synthesis and mitosis for expression of their differentiated characteristics (11–13), we assumed a similar hypothesis in the case of the Me\textsubscript{2}SO-induced differentiation of FLC.

In attempts to demonstrate a relationship between the induction of hemoglobin synthesis and cellular replication, cultures were treated with Me\textsubscript{2}SO under conditions that were either conducive or nonconducive to growth. Nonconducive conditions included seeding at high cell density, seeding in medium containing low serum, or adding N\textsubscript{6},O\textsuperscript{\textprime}dibutyryladenosine 3':5'-cyclic monophosphate (Bt\textsubscript{2}cAMP) to the culture medium.

The time course of hemoglobin accumulation was also studied in synchronous cultures in FLC. The results of these experiments indicate that two generations, after stimulation with Me\textsubscript{2}SO, are required before hemoglobin synthesis can be initiated.

MATERIALS AND METHODS

Cell Line. The Friend-virus-infected leukemic cells used in this study were derived from DBA/2 J mice (1, 4). This particular line (GM-86, clone 745) was obtained from the Mammalian Genetic Mutant Cell Repository, Institute of Medical Research, Capewood and Davis Streets, Camden, N.J. 08103.

The cells were routinely grown in Eagle's minimal essential medium (14) with Hanks' balanced salt solution (15) and 15% fetal calf serum. Penicillin (100 units/ml) and streptomycin (100 units/ml) were also added to the medium. The cultures were incubated in plastic flasks (Falcon) at 37\textdegree under a humidified atmosphere of 95% air–5% CO\textsubscript{2}.

Reagents. Reagent-grade dimethylsulfoxide (Mallinkrodt) was added, unsterilized, to the tissue-culture medium just prior to use. Bt\textsubscript{2}cAMP (sodium salt), purchased from Sigma Chemical Co., was dissolved in Puck's saline A (16) at 100 times the concentration desired in the medium.

Hemoglobin Assay and Cell Counting. Hemoglobin was measured spectrophotometrically at 410 nm. For these measure-
ments, 5-ml aliquots of a cell suspension were pelleted at 1000 \times g, washed twice with saline, lysed in 0.5 ml of distilled water, and frozen until the end of the experiment. After thawing, the lysates were cleared of debris by centrifugation (1500 \times g), and the supernatants were adjusted to 2% (w/v) in sodium dodecyl sulfate. The absorbance of the dodecyl-sulfate-treated lysates was measured at 410 nm. Linear extrapolation to baseline absorbance was used to determine the time of initiation of hemoglobin synthesis.

The number of cells per cm³ of suspension was determined by counting in a hemacytometer, using the average of two or more determinations.

Synchronization. FLC cultures were synchronized by seeding in isoleucine-deficient medium (17). Prior to seeding the cells were washed three times in isoleucine-deficient Eagle’s minimal essential medium supplemented with 15% dialyzed fetal calf serum. This method has been reported to block Chinese hamster ovary cells in the G₁ phase of the cell cycle. After 40 hr in the isoleucine-deficient medium, the cells were released by diluting the suspensions with normal medium and adjusting the isoleucine concentration to approximately normal levels with a 200X stock solution.

**RESULTS**

Requirement of Two Generation Times for the Induction of Hemoglobin Synthesis by Me₂SO. The time courses of cell growth and the appearance and accumulation of hemoglobin in FLC cultures, seeded at various densities, are shown in Fig. 1. In the absence of Me₂SO, negligible amounts of hemoglobin are made. Addition of Me₂SO (2% v/v) to the medium results in a 10-fold or greater stimulation of hemoglobin synthesis without any significant effect on cell growth.

When cultures are seeded at a low density (Fig. 1A), there is a 24-hr lag period before they enter the logarithmic growth phase. Hemoglobin synthesis appears to be initiated at 72 hr. Since the lag period extends for 24 hr, the period of cell division prior to hemoglobin synthesis is approximately 48 hr. At this time the cells are in log phase, and the cell number increases from 1.5 \times 10^6 to 5.6 \times 10^6 cells per cm³. This is approximately equivalent to two generations, and on the basis of these data we assign a mean generation time of 24 hr to these cells. These studies clearly indicate that most cells pass through two generations after the addition of the inductive stimulus (Me₂SO) before hemoglobin synthesis is initiated.

When cultures are seeded at a moderately high cell density (4.2 \times 10^6 cells per cm³) there is no lag period, and hemoglobin synthesis begins at about 48 hr (Fig. 1B). Again, the data show that the cells must go through two generations in the presence of Me₂SO before hemoglobin synthesis occurs. (In this case the cell number increased from 4.2 \times 10^6 to 16 \times 10^6 cells per cm³ in 48 hr.)

In contrast to the above observations, when cultures are seeded at a high cell density (37 \times 10^6 cells per cm³), so that cellular replication does not occur, hemoglobin synthesis is not detected (Fig. 1C). The data show that at this high cell density a stationary population of cells is maintained for a period of 72 hr, and no hemoglobin synthesis is induced during this time. We note here that, although cell death occurs after 72 hr, prior to this the stationary population appears normal, and this 72-hr period in Me₂SO is ample time for the induction to occur.

We conclude from these studies that the FLC require approximately two generations after the addition of the inductive stimulus (Me₂SO) for the initiation of hemoglobin synthesis.

The Effect of Reduced Serum Content on the Generation Time of FLC and the Induction of Hemoglobin Synthesis by Me₂SO. When FLC are grown in medium supplemented with less than 10% fetal calf serum, the generation time is extended. The generation time of cells grown in Eagle’s minimal essential medium +15% fetal calf serum is approximately 24 hr. When the serum content is reduced to 7.5% and 2.5%, the generation time is increased to 34 hr and 48 hr, respectively (Fig. 2A and B). If the stimulation of hemoglobin synthesis required two generations after the addition of Me₂SO, then the time of initiation of synthesis (as is indicated by the
extrapolated time) should be approximately the same as the time at which the cell population increased 4-fold. The data shown in Fig. 2 are in agreement with our hypothesis. In 2.5% fetal calf serum (Fig. 2A), the second generation is completed at about 96 hr, and the time of induction (as indicated by extrapolation) is at 96 hr. Similarly, in 7.5% fetal calf serum (Fig. 2B), the second generation is completed at 72 hr and hemoglobin synthesis begins at the same time, 72 hr. We conclude from these studies that two generations are required after the addition of Me2SO for the induction of hemoglobin synthesis.

The Effect of Bt2cAMP on the Generation Time of FLC and the Induction of Hemoglobin Synthesis by Me2SO. It has been shown that the generation time in a variety of cell lines is lengthened by Bt2cAMP (18-21; B. E. Ledford and J. Papaconstantinou, manuscript in preparation). In addition, intracellular cAMP levels have been shown to increase with cell density (22-24). In view of the above, cultures were treated with Bt2cAMP to determine whether the generation time of the FLC is altered and whether this, in turn, is reflected in the time of appearance of hemoglobin. Cultures were supplemented with Bt2cAMP at 24-hr intervals after seeding in Me2SO-containing medium (Fig. 3). Addition of Bt2cAMP (0.5 mM or 1.0 mM) at the beginning of the culture period resulted in complete inhibition of cellular replication. Under these conditions no hemoglobin synthesis occurs in Me2SO-treated cells. Addition of 1 mM Bt2cAMP to cultures 24 hr after the addition of Me2SO extended the generation time from 24 hr to approximately 38 hr and delayed the appearance of hemoglobin to 78 hr. In this case it can be seen that a time period equivalent to two generation times elapsed before hemoglobin synthesis appeared. In addition, only about 60% of the cells replicated two times before hemoglobin synthesis was initiated, and the rate of accumulation of hemoglobin was roughly 60% of the control level. Finally, when 1 mM Bt2cAMP was added to cultures after 48 hr (Fig. 3A), there was no effect on the time of appearance of hemoglobin even though cellular replication was significantly inhibited. In this case the cells replicated two times and although the Bt2cAMP inhibited further growth, hemoglobin synthesis was initiated after the second generation.

Induction of Hemoglobin Synthesis in Synchronized FLC. Synchronization of the Friend leukemia cells was achieved by the isoleucine deficiency method developed by Ley and Tobey (17). The cells were suspended in isoleucine-deficient medium for 40 hr to bring about cellular arrest at the G1 phase. The cells were released from the block by addition of isoleucine-containing medium, and Me2SO was added to induce hemoglobin synthesis. Hemoglobin appeared at 47 hr after the release (Fig. 4). During this time the cells went through two mitoses prior to the appearance of hemoglobin.

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**Fig. 2.** The effect of lower serum content on the generation time of Friend leukemia cells and on the induction of hemoglobin synthesis by Me2SO. (A) FLC in 2.5% fetal calf serum. (B) FLC in 7.5% fetal calf serum. ●, cells per cm² in Me2SO-containing medium; ▲, absorbance at 410 nm in Me2SO-containing medium.

**Fig. 3.** The effect of Bt2cAMP on the growth and time of hemoglobin synthesis. Cultures were seeded at 2.5 × 10⁶ cells per cm² in medium with or without 2% (v/v) Me2SO. Each point represents a 5-ml culture. At the time of seeding and at 24-hr intervals thereafter, the compound was added and appropriate bottles were removed for cell counts (A) and absorbance measurements at 410 nm (B). Since the growth curves with and without Me2SO were not sufficiently different, only the Me2SO curves are shown. The absorbance at 410 nm of lysates from cultures without Me2SO are subtracted from the values for the corresponding Me2SO-treated cells. ●, no additions; ▲, 0.5 mM Bt2cAMP added at time 0; ■, 1 mM Bt2cAMP added at time 0; ○, 1 mM Bt2cAMP added at 24 hr; △, 1 mM Bt2cAMP added at 48 hr; □, 1 mM Bt2cAMP added at 72 hr.
DISCUSSION

An essential regulatory event in the sequence of stages leading to the differentiation of specific tissues and cell types, and to the expression of tissue-specific biochemical functions, is the requirement for a critical number of cell replications in the presence of a specific inductive stimulus. These phenomena of obligatory DNA synthesis and critical mitosis have been described for the erythropoietin stimulation of hemoglobin synthesis in fetal mouse liver (13) and in chick embryo erythroblasts (11), and for the prolactin stimulation of milk protein synthesis in mouse mammary gland explants (12). In all three cases DNA synthesis and/or mitosis, in the presence of the hormone, is critical for further cell differentiation. FLC derived from mouse spleen respond to MeSO by synthesizing hemoglobin and acquiring other characteristics of differentiating erythroid cells (5). In these studies we have been able to show that in the presence of MeSO, which is not a physiological inducer, these cells must go through two rounds of DNA synthesis and/or mitosis before hemoglobin synthesis is initiated. Thus, we conclude that this model system can mimic erythropoietin with respect to the role of critical mitosis in the induction of hemoglobin synthesis.

Although it is not definitive, the requirement for DNA synthesis in erythropoiesis and mammary gland differentiation is implied by the inability of the cells to initiate the synthesis of their respective specific proteins in the presence of BrdUrd (11, 25). At present, there is no direct evidence from any of these systems that incorporation of BrdUrd into specific sites in the genome results in the inhibition of synthesis of a specific protein. Scher et al. (4) have shown that the response of FLC (clone 745) to MeSO can be inhibited up to 70% by 10 μM BrdUrd if the analog is administered simultaneously with the MeSO. If the BrdUrd is administered 2 days after the addition of MeSO, the effect of the inhibitor on hemoglobin synthesis is greatly reduced, and if it is administered after 3 days, hemoglobin synthesis is not affected. Thus this loss of sensitivity to BrdUrd occurs after the cells have divided twice in the presence of the inducer, further implicating the requirement for DNA synthesis in this induction. In addition, it appears thus far that the MeSO induction and the naturally occurring hormonal induction are very similar in their timing, their requirement for DNA synthesis, and their inhibition by BrdUrd. The strongest evidence for the requirement of DNA synthesis in the induction by MeSO is presented by the studies in which BrdUrd-resistant clones of FLC were used (26).

In these studies it was shown that the extent of BrdUrd incorporation is greatly reduced in the resistant strain, and that the extent of inhibition of hemoglobin synthesis is equally reduced. Thus the extent of inhibition of hemoglobin synthesis is related to the extent of incorporation of BrdUrd.

In these studies we have presented the following evidence supporting the hypothesis that FLC require two rounds of DNA synthesis and/or mitosis after the addition of MeSO for the induction of hemoglobin synthesis: (a) In asynchronous cultures, the time of onset of detectable hemoglobin synthesis corresponds to the length of time required for the cell number to increase 4-fold. (b) Cells grown in the presence of MeSO, but under conditions where growth is prevented [i.e., by a high population density or by high concentration of Bt2cAMP] do not synthesize hemoglobin. (c) If the generation time of FLC is increased by treatment with lowered serum content or with Bt2cAMP, the time of initiation of hemoglobin synthesis is delayed until two generations have been completed. (d) Hemoglobin synthesis in synchronous cultures is initiated after the second wave of mitosis. We conclude that two rounds of DNA synthesis are required for the MeSO-induced expression of differentiated function of FLC.

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