The Role of Heme in the Maturation of Erythroblasts: The Effects of Inhibition of Pyridoxine Metabolism

By Leslie M. Hoffman and Jeffrey Ross

The role of heme in the maturation of erythroblasts and in globin gene expression was investigated with two in vitro erythroid cell culture systems, T2-C12 murine erythroleukemia cells (MELC) and primary cells from mouse fetal liver (MFL). The accumulation and/or rates of synthesis of globin protein, heme, and globin mRNA were assayed in cells in which endogenous heme biosynthesis was reduced by the tuberculostatic drug isonicotinic acid hydrazide (INH). Under conditions in which heme synthesis was reduced 2–4-fold in dimethylsulfoxide (Me2SO) treated MELC and 5–10-fold in MFL erythroblasts, the levels of globin protein were reduced 10-fold and 2-fold, respectively. The rates of synthesis of globin protein were reduced to similar extents without a concomitant decrease in total protein synthesis. The levels of globin mRNA in INH-treated MELC and MFL cells were 2–7-fold lower than those in untreated cells. In MELC cells cultured with both INH and hemin, the globin protein and mRNA levels were the same as in cells cultured with hemin alone. The globin mRNA content of cells cultured with Me2SO plus INH was low but increased within 5 hr after the addition of hemin. Thus, hemin overcame the inhibitory effects of INH. These data are consistent with the hypothesis that heme affects globin gene expression at the pretranslational, as well as the translational level.

THE PRODUCTION of erythrocytes from undifferentiated stem cells must occur in a highly regulated manner, so that a relatively constant number of erythrocytes is maintained in the organism. The differentiation process involves three major steps: commitment of the stem cell to the erythroid pathway, development of immature “burst-forming” and “colony-forming” erythroid precursor cells to proerythroblasts, and last, end-stage development, involving hemoglobinization and (in mammals) enucleation of erythroblasts. The factors that regulate the commitment of stem cells to erythroid burst-forming and colony-forming cells are unknown. The glycoprotein hormone erythropoietin is required for the maturation and/or replication of colony-forming cells to proerythroblasts. It is not clear to what extent, if any, erythropoietin is required for the subsequent maturation of proerythroblasts to mature erythrocytes. However, it is reasonable to propose that the final stages of erythroid differentiation, like the early stages, are stringently regulated by one or more factors. It is unlikely that homeostasis is maintained by erythropoietin alone.

The experiments reported in this article concern the potential regulatory role of heme in proerythroblast maturation. Heme is known to play at least two roles in erythrocyte biology: (1) it is a structural component of hemoglobin and (2) it regulates the expression of globin messenger RNA at the translational level. We propose that heme plays a third role, separate and distinct from these two, namely, that it regulates the rate of maturation of proerythroblasts to erythrocytes. The regulatory function of heme might be trophic, involving the expression of a set of erythroid-specific genes, or it might be specific, involving the induction of globin gene transcription.

The most direct experimental evidence in support of this hypothesis comes from experiments with Friend murine erythroleukemia cells (MELC). Hemin is a potent inducer of MELC maturation. Within 4 hr after the addition of hemin to T2-C12 MELC, the content of globin messenger RNA increases significantly above that of control cells, implying that heme or a heme metabolite induces globin gene transcription. Less direct evidence is derived from observations with experimental animals and man. Animals in whom the utilization of iron and protoporphyrin IX is not properly balanced may develop sideroblastic anemia, characterized by a reduction in the number of circulating erythrocytes, the presence of sideroblasts (immature erythroid cells whose mitochondria contain excess nonheme iron), and hypochromic erythrocytes. Sideroblastic anemias occur in mutant “flexed” mice and in animals whose diet is deficient in pyridoxine, which is required for heme synthesis. In man, sideroblastic anemia may be inherited, acquired, or induced by agents that block heme synthesis, including ethyl alcohol and the tuberculostatic drug, isonicotinic acid hydrazide (INH).

To investigate the potential role of heme in late stage erythroblast maturation, we have studied the effect of a heme biosynthesis inhibitor on the maturation of MELC and of primary erythroblasts from mouse embryonic liver. The goal of these studies was to reduce the capacity of these cells to synthesize heme...
without significantly affecting their viability or their ability to replicate. An adequate supply of heme is required for cell viability, because heme is a constituent of a number of enzymes and a cofactor required for oxidative phosphorylation. Therefore, it was necessary to define conditions in vitro (in tissue culture) that mimic those in sideroblastic anemia in vivo. That is, heme synthesis must be reduced, but not completely inhibited. We describe cell culture conditions that meet these criteria, and we present evidence that the late stage erythroblast is unable to mature efficiently when its capacity to accumulate heme is impaired. The data are consistent with the hypothesis that heme directly affects globin gene transcription, perhaps at the transcriptional level.

MATERIALS AND METHODS

Cell Culture and Immune Hemolysis of Fetal Liver Hemoglobinized Erythroblasts

Murine erythroleukemia cells (T3-Cl2) were maintained in Ham F12 medium containing 10% fetal calf serum (FCS, Gibco, Grand Island, N.Y.) as previously described.4 Inducers were added 4 hr after logarithmically growing cells were subcultured into fresh medium at 5 × 10^6 cells/ml, unless otherwise noted. INH (Sigma, St. Louis, Mo.) was dissolved in Ham F12 medium and filtered through a 0.2-μm membrane. Hemin (Sigma, bovine) was dissolved according to Freedman et al.18 and was sterilized by filtration prior to addition to the culture medium. Unfiltered Me3SO (Schwarz-Mann) was added directly to the cultures. Viable cells were counted in a hemacytometer in the presence of trypan blue.

Erythroid cells from the livers of 13- or 14-day-old embryonic mice were dissected as previously described15 and were placed in Ham F12 medium containing 10% FCS, 50 U penicillin, and 50 μg streptomycin/ml (growth medium). Immune hemolysis of hemoglobinized erythroblasts was performed essentially as described by Cantor et al.15 This procedure was used to prepare nonhemoglobinized proerythroblasts and basophilic erythroblasts from the mixed population of hemoglobinized and nonhemoglobinized erythroblasts of the fetal liver. The cells are incubated with rabbit antiserum raised against mouse red blood cells and with guinea pig complement, and late-stage cells are selectively lysed. Briefly, fetal liver cells were suspended at 6 × 10^6 cells/ml in 100 ml growth medium in a spinner flask. Rabbit antiserum to mouse erythrocytes, diluted 1/400-1/500, and guinea pig complement (Gibco), reconstituted and Sautner.1 The minimum quantity of hemoglobin detectable by this method is 0.2 g.

Heme Synthesis

Heme synthetic capacity was assayed by measuring the incorporation of 59Fe into heme. One milliliter of 59FeCl3, 5 mCi/mg, 0.1 mCi/ml (Amersham/Searle), was added to 0.5 ml of unbuffered 3.46 mM sodium citrate. This mixture was added dropwise to a fivefold excess (v/v) of FCS and incubated for 1 hr at 37°C, to allow for iron binding to transferrin.30 The FCS-59Fe was then added to a final concentration of 1.5 μCi/ml and 10% FCS to cells suspended in Ham F12 medium. At the end of the labeling period, cells were collected by centrifugation and washed 4 times at 4°C with iron-free F12 medium. An aliquot was removed prior to the final centrifugation to obtain a cell count. Cell lysis was accomplished by the addition of 3 ml distilled water, followed by 3 cycles of freezing in an ethanol-dry-ice bath and thawing at 37°C. Total iron uptake was determined by counting an aliquot of the lysates in a gamma counter.

Hemin was extracted into cyclohexanone according to Teale11 at room temperature. Control experiments demonstrated that less than 0.01% of the radioactivity of aqueous 59FeCl3 was extracted into the cyclohexanone phase under these conditions. The total iron content of erythroleukemia cells was determined by a modification of the method of Ramsay.22 A known quantity of cells was processed exactly as per the 5FeCl3-treated cells, except that nonradioactive FeCl3 was added as the Fe-transferrin complex. The cells were harvested, washed with iron-free F12 medium, and lysed by freeze-thawing in 0.25 ml of H2O. To the lysate (0.25 ml) were added equal volumes of freshly prepared 0.1 M Na2SO3 and 0.1% (v/v) dipryridyl in 3% acetic acid. The mixture was heated in a boiling water bath for 5 min, cooled to room temperature, and 1 ml chloroform was added. After mixing thoroughly for 1 min, the mixture was centrifuged at 2000 rpm in the PR J centrifuge at room temperature for 10 min. The aqueous supernatant was removed and reextracted with 0.5 ml chloroform. The A_59 of the supernatant was measured in a Cary model 118 spectrophotometer. Appropriate concentrations of FeNH4(SO4)_2 · 12H2O were used as standards, and absorbance was linear with iron concentration to a maximum of 10 μg/ml.

Steady-State Level of Globin Protein

Globin protein was determined by Ouchterlony immunodiffusion of cytoplasmic lysates to an endpoint dilution, as described by Ross and Sautner.4 The minimum quantity of hemoglobin detectable by this method is 0.2 μg.

Total Protein and Globin Synthesis

Friend cells were harvested 4 days after subculturing and were resuspended in 10 ml of growth medium containing appropriate additions plus 500 μCi of 1-4,5-3H-leucine (Amersham/Searle,
48–60 Ci/mmmole). After 30 min at 37°C, the cells were harvested, washed twice in F12 medium, and lysed with 2.5 ml of 0.5% Triton X-100 in NKM (0.13 M NaCl, 0.5 mM KCl, 7.5 mM MgCl₂). Lysates were centrifuged at 5000 rpm in a Sorvall SS-34 rotor at 4°C for 15 min. To 0.9-ml aliquots of the supernatant were added 10 μg ovalbumin (Miles, 5 times recrystallized) and 30 μl rabbit anti-ovalbumin serum (Miles). The mixture was incubated at 37°C for 1 hr and then at 4°C overnight. The precipitate was removed by centrifugation, and 15 μg of 14C-mouse globin, an internal control to monitor the efficiency of immune precipitation, 0.2 ml of sheep antibody serum or control sheep serum, and 0.3 ml of 0.1 M NaCl were added to 0.2 ml of the supernatant. After incubation at 37°C for 1 hr and 4°C overnight, the precipitate was collected by centrifugation and washed 4 times with 0.14 M NaCl. 14C-mouse globin was added to samples incubated with control serum. Immune precipitates were dissolved in 3% SDS, 10% glycerol, 0.0624 M Tris-Cl, pH 6.8, and were electrophoresed in SDS-15% polyacrylamide stacking slab gels as described by Laemmli.23 Gels were stained with Coomassie brilliant blue, the globin bands cut out, eluted and counted in 10 ml toluene/PPO scintillant. To correct for the recovery of 1H-globin radioactivity, the 3H disintegrations per minute (dpm) of each globin band was divided by the efficiency of recovery of the 14C-globin internal standards added before immune precipitation or before electrophoresis.

Mouse fetal liver cells were labeled with 3H-leucine as described for Friend cells. They were lysed by the addition of 1 vol of 1.5 M MgCl₂, mixed thoroughly, diluted with 3 vol of 0.01 M Tris-Cl, 0.01 M KCl, 0.5 mM MgCl₂, pH 7.5, and centrifuged at 10,000 rpm in a Sorvall RC2-B rotor at 4°C for 20 min. The supernatant was centrifuged at 105,000 g in a Spinco SW-60 rotor in a Beckman L2-65-B ultracentrifuge at 4°C for 90 min. The hemoglobin in the supernatant was analyzed by isoelectric focusing in 4% polyacrylamide gels containing 5% glycerol and 1% each 3–10 and 5–7 Biolyte ampholytes (Bio-Rad), essentially as described by Righetti and Drysdale.24 The hemoglobin bands were identified by coelectrophoresing freshly prepared CD-1 mouse erythrocyte lysate. The portion of the gel containing hemoglobin was cut out, eluted, and counted exactly as described above for the analysis of globin in SDS gels.

Total protein synthesis was assayed by precipitating duplicate 0.1-ml aliquots of cell lysates with 0.1 ml 10% trichloroacetic acid (TCA). Precipitates were heated to 90°C for 15 min to degrade aminoacyl tRNA, cooled to 0–4°C, filtered onto GF/C filters (Whatman), incubated for 15 min with 1 ml NCS:H₂O (5:1), and counted in 10 ml toluene/PPO scintillant. To correct for the recovery of 1H-globin radioactivity, the 3H disintegrations per minute (dpm) of each globin band was divided by the efficiency of recovery of the 14C-globin internal standards added before immune precipitation or before electrophoresis.

RNA Isolation and Hybridization Assay

Total erythroblast RNA was extracted from urea-SDS lysates with phenol-chloroform and pelleted through CsCl as described by Ross.35 T₃-Cl₂ cell RNA was isolated from Sarkosyl lysates as previously described. Unlabeled globin mRNA was assayed by solution hybridization,3 and radioactive globin RNA was assayed by solution hybridization with unlabeled cDNA.35

RESULTS

Effects of INH on Erythroid Cell Growth, Viability, Heme Synthesis, and Globin Protein Accumulation

INH inhibits several pyridoxal phosphate-dependent enzymes in the heme biosynthetic pathway, including delta-aminolevulinic acid synthetase.29 It seemed likely that INH would inhibit heme synthesis in erythroid cells as well, but it was necessary to titrate the INH to levels that would reduce heme synthesis but not be cytotoxic. Low concentrations of INH (1–4 mM) had little or no effect on the growth of murine erythroleukemia cells (MELC), but higher concentrations were inhibitory (Fig. 1A). Cells treated with Me₂SO plus INH reached a lower saturation density than cells cultured in Me₂SO alone (Fig. 1B, open and closed circles), while cells cultured with hemin plus INH achieved a maximum density similar to that of cells cultured with hemin alone (Fig. 1B, open and closed triangles). INH at low levels did not induce T₃-Cl₂ cells to differentiate (see Tables 3, 5 and 8). Therefore, 4 mM INH was chosen as the optimal concentration for experiments with MELC.

Nonimmune hemolyzed mouse fetal liver (MFL) cells were cultured for 24 hr in the presence of erythropoietin (EPO, human urinary) and various concentrations of INH. At the INH concentrations chosen for subsequent experiments (8–10 mM), cell viability was only slightly reduced, as compared with control cultures (Fig. 1C).

The effect of INH on heme biosynthesis was measured at early times during the maturation process, prior to the time of maximal hemoglobinization. To assess the effects of reduced heme synthesis on globin gene expression, it was necessary that the inhibitor function in nonhemoglobinized cells, because heme deprivation during the final maturation stages would directly reduce intracellular hemoglobin content. The incorporation of ⁵⁷Fe into heme in MFL erythroblasts and in T₃-Cl₂ cells was assayed in the presence or absence of INH. The intracellular iron pool and the specific activity of the ⁵⁷Fe were determined, so that heme synthesis could be measured independent of Fe uptake. Heme synthesis in MELC cultured with INH plus Me₂SO was reduced by 30% as early as 3 hr after INH addition, compared to cells receiving Me₂SO alone (Table 1, experiment 1, column 8). The extent of inhibition was 44% by 24 hr (Table 1, experiment 2, column 8). Since globin mRNA accumulation in Me₂SO-treated T₃-Cl₂ MELC does not begin until 36 hr after Me₂SO addition (Ross et al., 1972),25a we conclude that INH reduces heme synthesis prior to the onset of globin mRNA accumulation. ⁵⁷Fe incorporation in MELC receiving hemin (10⁻⁴M) or hemin plus INH (4 mM) was not detectable at 24 hr (data not shown).

Immune hemolyzed MFL cells were cultured for 2 hr with or without 10 mM INH and were then incubated with ⁵⁷Fe for an additional 2 hr. INH inhibited ⁵⁷Fe uptake by approximately 50%, and it reduced ⁵⁷Fe incorporation into heme by 83%–90% (Table 2). We conclude that INH reduces heme...
ROLE OF HEME IN ERYTHROBLAST MATURATION

Fig. 1. The effect of INH on cell growth and viability. INH was added to erythroleukemia cells at the time of subculture. Total mouse fetal liver cells (not immune-lysed) were cultured for 24 hr with 0.15 U/ml EPO and the indicated concentrations of INH. After harvesting and washing with F12 medium, the viable cells were counted in a hemocytometer in the presence of trypan blue. (A) Erythroleukemia cells cultured with or without INH. No additions, ○; 1 mM INH, △; 2 mM INH, □; 4 mM INH, ●; 10 mM INH, Δ; 50 mM INH. (B) Erythroleukemia cells cultured with or without INH in the presence of Me2SO and/or hemin. No additions, ■; 1.5% Me2SO. ○; 1.5% Me2SO plus 4 mM INH, ●; 10⁻⁴ M hemin, △; 10⁻⁴ M hemin plus 4 mM INH, A. (C) Mouse fetal liver cells cultured for 24 hr with or without INH.

synthesis in MFL erythroblasts before they have reached that stage of maturation in which globin gene expression is maximal.²⁷

These results encouraged us to analyze the effects of INH on globin protein accumulation and synthesis. MELC cultured for 5 days were harvested, washed extensively, and stained with dimethoxybenzidine, which reacts with the heme moiety of hemoglobin.¹⁹ Cultures incubated with Me₂SO contained 36% benzidine positive (B⁺) cells, as compared with 8% B⁺ cells in cultures treated with Me₂SO plus INH (Table 3). Cultures with no additions or with INH (4 mM) had no detectable B⁺ cells.

The accumulation of globin protein was measured by a semiquantitative immunoprecipitation assay. INH treatment resulted in a tenfold reduction in
of labeling, the erythroblasts, initially 2%-17% benzidine positive, cultures incubated with nonradioactive FeCl₃ were analyzed for total cell Fe by a modification of the method of Ramsay. Specific activities of nonheme 24 hr, the cells were harvested and resuspended in 6,787 23. Biosynthesis.

Table 1. Inhibition by INH of Fe Incorporation Into Heme in Me₂SO-Treated Erythroleukemia Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time in Culture (hr)</th>
<th>Additions</th>
<th>Total Cellular Fe Incorporation (cpm/10⁷ Cells)</th>
<th>Heme Synthesis (cpm/10⁷ Cells)</th>
<th>Total Cellular Iron Content (µg/10⁷ Cells)</th>
<th>Specific Activity of Intracellular Fe (cpm/µmole)</th>
<th>Heme Synthesis (µmole) 10⁷ Cells/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Me₂SO</td>
<td>3.3 x 10⁶</td>
<td>577</td>
<td>1.80</td>
<td>1.0 x 10⁴</td>
<td>190</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Me₂SO + INH</td>
<td>2.7 x 10⁴</td>
<td>438</td>
<td>1.35</td>
<td>1.1 x 10⁴</td>
<td>133</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>None</td>
<td>2.0 x 10⁴</td>
<td>615</td>
<td>1.33</td>
<td>1.1 x 10⁴</td>
<td>180</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>Me₂SO</td>
<td>2.4 x 10⁴</td>
<td>559</td>
<td>0.63</td>
<td>2.1 x 10⁴</td>
<td>90</td>
</tr>
<tr>
<td>24</td>
<td>Me₂SO + INH</td>
<td>1.7 x 10⁴</td>
<td>376</td>
<td>0.35</td>
<td>2.5 x 10⁴</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

*These data represent the average of two samples.

T₃-C₁₂ cells were subcultured to fresh medium and were incubated with 1.5% Me₂SO, 1.5% Me₂SO plus 4 mM INH, or with no additions. After 3 or 24 hr, the cells were harvested and resuspended in F12 medium containing the appropriate additions, and transferrin-bound Fe was added. After 3 hr of labeling, the total cellular uptake of Fe and the incorporation of Fe into heme were determined as specified in "Experimental Procedures." Parallel cultures incubated with or without FeCl₃ were analyzed for total cell Fe by a modification of the method of Ramsay. Specific activities of nonheme Fe were calculated as follows:

\[
\text{cpm Total cell }^{56}\text{Fe} \times \frac{\text{cpm Heme }^{56}\text{Fe}}{10^7 \text{Cells}} \times \frac{55.85 \mu\text{mole Fe}}{\mu\text{mole Fe}} = \text{cpm/µmole Fe}
\]

Hemoglobin content in Me₂SO-treated MELC (Table 3). These results suggest that INH affects the hemoglobin content of erythroblasts by interfering both with heme and globin protein synthesis (see following section). If the reduction of globin protein and globin mRNA (see following section) by INH was the result of inhibition of endogenous heme synthesis, then the addition of exogenous hemin to the culture medium of INH-treated cells should overcome the inhibitory effect. Hemin was added to the medium of MELC cultured with or without INH, and the quantities of hemoglobin per cell were essentially the same in each case (Table 3). These data demonstrate that exogenous hemin overcomes the effect of INH on globin protein accumulation in MELC and provides assurance that the primary effect of INH is related to heme biosynthesis.

MFL cells that are stained by dimethoxybenzidine have made the transition between basophilic and polychromatophilic erythroblasts. Immune-hemolysed erythroblasts, initially 2%-17% benzidine positive, were cultured with or without INH in the presence of EPO. After 24 hr, the cells were centrifuged onto microscope slides and stained. Cultures treated with INH contained an average of 42% fewer B⁺ cells than control cultures, and the globin protein content was reduced by 50% (Table 4). Although exogenous hemin affects globin gene expression dramatically in MELC⁺

Table 2. Incorporation of Fe Into Heme in Fetal Liver Erythroblasts Incubated With or Without INH

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Total Cellular Fe (cpm/10⁷ Cells) x 10⁻¹⁴</th>
<th>Heme Fe (cpm/10⁷ Cells) x 10⁻¹⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>None</td>
<td>61.3 (100)</td>
<td>11.7 (100)</td>
</tr>
<tr>
<td></td>
<td>INH</td>
<td>28.8 (47)</td>
<td>1.1 (9)</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>137.9 (100)</td>
<td>17.1 (100)</td>
</tr>
<tr>
<td></td>
<td>INH</td>
<td>85.8 (62)</td>
<td>2.9 (17)</td>
</tr>
</tbody>
</table>

Immune-lysed cells were incubated for 2 hr with or without INH (10 µM) prior to the addition of Fe. The labeling period was 2 hr. Numbers in parentheses are the percent of control values for each experiment.

*These data represent the average of duplicate samples.

Table 3. Heme and Globin Accumulation in Erythroleukemia Cells

<table>
<thead>
<tr>
<th>Additions</th>
<th>Benzidine-Positive Cells (%)</th>
<th>Globin (µg/Cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;1</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>INH</td>
<td>&lt;1</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Me₂SO</td>
<td>36</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Me₂SO + INH</td>
<td>8</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Hemin</td>
<td>16</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Hemin + INH</td>
<td>23</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

T₃-C₁₂ cells were cultured for 5 days, harvested, washed, and an aliquot was removed for benzidine staining. Additive concentrations were: Me₂SO, 1.5% (v/v); hemin, 10⁻² M; INH, 4 mM. Hemoglobin was quantitated by the double immunodiffusion assay. Data are the mean and standard error of the mean for 3–5 separate experiments.

Table 4. Heme and Globin Accumulation in Fetal Liver Erythroblasts Cultured for 24 hr With or Without INH

<table>
<thead>
<tr>
<th>Immune Hemolysis</th>
<th>Treatment</th>
<th>Benzidine-Positive Cells (%)</th>
<th>Globin (µg/Cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>None, zero time</td>
<td>77 (104)</td>
<td>6.3 (107)</td>
</tr>
<tr>
<td>+</td>
<td>None, zero time</td>
<td>11* (15)</td>
<td>1.6 (27)</td>
</tr>
<tr>
<td>+</td>
<td>None, 24 hr</td>
<td>74* (100)</td>
<td>5.9 (100)</td>
</tr>
<tr>
<td>+</td>
<td>INH, 24 hr</td>
<td>43* (58)</td>
<td>2.9 (49)</td>
</tr>
</tbody>
</table>

MFL cells were cultured with or without 10 mM INH for the designated periods. Benzidine staining and hemoglobin content were determined as described in Materials and Methods. Numbers in parentheses are the percent of 24-hr untreated control values.

*These numbers are average of six separate experiments.
erythroblasts are morphologically immature, they are or globin protein in MFL erythroblasts (see Table 10 of globin mRNA translation, increased turnover of

to some extent programmed to become hemoglobinized.

Effects of Reduced Heme Synthesis on Total Protein and Globin Protein Synthesis

The reduction of globin protein accumulation could be the result of decreased globin mRNA, a slower rate of globin mRNA translation, increased turnover of
globin protein, or a combination of the above. To distinguish among these alternatives, the approximate rates of globin protein synthesis and turnover and the levels of globin mRNA (next section) were compared in treated and untreated cells. MELC on day 4 of subculture were incubated for 30 min with H-leucine, and cytoplasmic lysates containing authentic 14C-mouse globin as carrier were incubated with sheep anti-mouse globin serum or control sheep serum. After immunoprecipitation, 14C globin was added to the control serum samples, and the precipitates were dissolved and electrophoresed in SDS-polyacrylamide slab gels. 3H radioactivity in the globin band was corrected for the recovery of carrier 14C-globin, and the 3H radioactivity in the globin region of control serum precipitates was subtracted. Globin synthesis accounted for 2.2% of total protein synthesis in MELC on day 4, but only 0.5% in cells treated with MeSO and INH (Table 5). This inhibition was apparently quite specific for globin, since total protein synthesis was moderately elevated in INH-treated cells. Similar results were obtained in the MFL culture system, in which newly synthesized hemoglobin was assayed by isoelectric focusing. The percentage of hemoglobin synthesis was reduced by 40%–50% in INH-treated, as compared with control erythroblasts (Table 6).

These data indicate that INH specifically retards the rate of globin mRNA translation, perhaps by interfering with heme production. This hypothesis
is supported by the fact that the rate of globin synthesis in hemin-treated MELC is unaffected by INH (Table 5). That is, exogenous hemin reverses the inhibitory effect of INH.

The effect of INH on globin protein turnover was evaluated in MFL cells, which were incubated for 30 min with \(^3\)H-leucine and chased for 21 hr with growth medium containing nonradioactive leucine. Although the turnover of total protein in INH-treated MFL cells is similar to that of control cells (Table 7, column 2), hemoglobin turnover is 47% greater in INH-treated cells (Table 7, column 5). Therefore, hemoglobin synthesis and turnover are both affected by reducing heme synthesis in erythroblasts. This result is not surprising, in view of previous findings that free globin chains are unstable.

Effects of Decreased Heme Synthesis on Globin mRNA Accumulation

The inhibition of hemoglobin synthesis in INH-treated cells might arise entirely from the slower rate of globin mRNA translation as a result of reduced intracellular heme concentrations. However, if heme or a heme metabolite is involved directly in globin gene transcription, as was suggested by previous studies with MELC, we would predict that the accumulation of globin mRNA would also be affected by INH treatment. To determine if heme affects globin synthesis at the pretranslational as well as the translational level, the accumulation of globin mRNA was measured in the presence and absence of INH.

MELC were cultured with or without INH, hemin, Me\(_2\)SO, or a combination thereof for 5 days, at which time the cells were harvested, and total cell RNA was prepared. The RNA was incubated in solution with highly radioactive globin \(^3\)H-cDNA. This assay is highly specific for globin-specific RNA and is independent of its translatability. Cells cultured with Me\(_2\)SO alone contained almost sevenfold more globin mRNA than cells grown in the presence of Me\(_2\)SO plus INH (Table 8). In contrast, INH failed to affect the level of globin mRNA in hemin-treated cells, the globin mRNA content being even higher in the INH-treated cells. Similar results were again obtained in the MFL system. Erythroblasts cultured in the presence of INH contained 40% less globin mRNA, as compared with control cells (Table 9).

The responses of MELC versus MFL cells to INH differ quantitatively, although not qualitatively. In both cases, the quantity of globin mRNA is lower in INH-treated cells, but the levels of inhibition are sevenfold as compared to less than twofold in MELC versus MFL, respectively (Tables 8 and 9). Perhaps endogenous heme pools are higher in MFL erythroblasts than in MELC, so that the INH-induced reduction of heme synthesis (Table 2) exerts a relatively smaller effect in MFL cells. It is clear that exogenous hemin fails to amplify globin mRNA production in MFL cells, which are already producing significant quantities of globin mRNA, as demonstrated by the following experiment: Fourteen-day-old MFL cells (not immune-lysed) were placed in culture in the presence or absence of \(10^{-4}\)M or \(10^{-3}\)M hemin. After 90 min, \(^3\)H-nucleosides were added, and the cells were incubated for an additional 2 hr, at which time they were harvested and total cell RNA was prepared. The RNA was hybridized in solution with excess unlabeled globin cDNA to detect newly synthesized (radioactive) globin mRNA sequences.

As shown in Table 9, exogenous hemin does not significantly affect the quantity of globin mRNA synthesized during the 2-hr labeling period. This result differs from that observed in the MELC system, in which globin mRNA levels rise as early as 1 hr after hemin is added to the cells.

The experiments concerning the reduction of globin mRNA in INH-treated MELC (Table 8) are consistent with the hypothesis that heme affects erythroblast maturation, at least in part, at the pretranslational level. If this were the case, then we would predict that the addition of exogenous hemin to INH-treated cells would stimulate globin mRNA production. To test...
this hypothesis, MELC were cultured in the presence of Me2SO and INH for 42 hr. At that time, the cells were distributed into two flasks, one of which received 10^{-4} M hemin. Thus, one flask contained Me2SO plus INH, the other Me2SO plus INH plus hemin. At 5 and 30 hr thereafter, aliquots of cells were removed and total cellular RNA was prepared. Ten micrograms of RNA were incubated with globin 3H-cDNA, and the percentage of hybridization was determined. The results indicate that hemin rapidly induces the accumulation of globin mRNA in cells pretreated with Me2SO and INH (Fig. 2). It is important to note that the mechanisms of action of Me2SO and of hemin in the induction of MELC maturation are unknown. Therefore, experiments in which both inducers are used simultaneously must be interpreted with some caution. Nevertheless, the data of Fig. 2 do indicate that the effect of INH on globin mRNA accumulation in Me2SO-stimulated MELC is rapidly overcome by exogenous hemin.

**DISCUSSION**

The major conclusion from these experiments is that globin mRNA accumulation is inhibited under conditions in which heme synthesis is partially repressed. Our results support, but do not prove, the hypothesis that heme or a heme metabolite is required for globin gene transcription. In order to prove this hypothesis, it must be demonstrated that heme activates globin gene transcription in an in vitro system (in progress). Furthermore, experiments with inhibitors must be interpreted with caution, since few inhibitors are absolutely specific. The predominant effect of INH in MELC and MFL cells does appear to involve hemoglobin synthesis (Tables 1, 2, 5, and 6), and the inhibition of heme synthesis by INH in other erythroid cell culture systems is well documented. More importantly, the inhibitory effects of INH in MELC are specifically reversed by exogenous hemin (Tables 3, 5, and 8 and Fig. 2). Preliminary experiments also indicate that exogenous pyridoxine (1–5 × 10^{-5}M) completely reverses the inhibition of benzidine-positive MELC production by 10^{-5} M INH (L. M. Hoffman, unpublished observations).

If heme is required for globin gene transcription, then Me2SO might function either by elevating intracellular heme levels or by redistributing endogenous heme to different sites within the immature cell. These experiments support this possibility, because INH retards heme synthesis during early times after the addition of Me2SO, prior to the actual onset of globin mRNA accumulation. However, it is not clear whether all MELC lines follow such a temporal sequence, in which accelerated heme synthesis precedes globin mRNA accumulation (compare references 32–34). Furthermore, there is some genetic evidence that the induction of large quantities of heme and of globin mRNA are separable.

The use of INH with the established erythroleukemia cells might provide a useful in vitro tissue culture model system for investigating erythroid development in sideroblastic anemias. Globin protein synthesis is abnormal in the reticulocytes of some humans with congenital or idiopathic sideroblastic
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The role of heme in the maturation of erythroblasts: the effects of inhibition of pyridoxine metabolism

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