The Relationship of Hemoglobin Synthesis to Erythroid Colony and Burst Formation

By James F. Eliason, Gary Van Zant, and Eugene Goldwasser

We have demonstrated that the cyclohexane-one method for the extraction of hematin can be used to measure hemoglobin synthesis induced by erythropoietin (epo) in mouse bone marrow cells cultured in medium containing methyl cellulose. The time course of hemoglobin synthesis by mouse marrow cells showed two effects due to epo: an increase in hemoglobin synthesis at day 2, which corresponded to the formation of small erythroid colonies resulting from the CFU-E (colony-forming unit, erythroid), and a very large increase in hemoglobin synthesis, which was maximal at days 7–8 and corresponded to the formation of large erythroid colonies (bursts) resulting from the BFU-E (burst-forming unit, erythroid). The epo dose–response curves for CFU-E colony counts and day-2 hemoglobin synthesis were similar, and the cell-number–response curves for these two parameters were parallel. The epo dose–response curve for BFU-E colony counts reached a plateau at an epo concentration between 3 and 5 units/ml, whereas the dose–response curve for 6–8-day hemoglobin synthesis did not reach a plateau even at an epo dose of 10 units/ml.

Ever since Stephenson et al. first reported that mouse fetal liver cells could be stimulated by erythropoietin (epo) to form small colonies of hemoglobin-containing cells in plasma clot cultures there has been much interest in erythroid colonies and their progenitor cells, the CFU-E (colony-forming unit, erythroid). Assay systems have been developed for detection of CFU-E from a number of different species, including the rat, man, sheep, goat, and chicken.

A second type of erythroid progenitor was detected after 7 days of incubation in cultures of mouse bone marrow cells that had been either fed repeatedly with epo or given a single high dose of epo. This progenitor cell, called the BFU-E (burst-forming unit, erythroid), gives rise to very large diffuse colonies called bursts. The BFU-E have been detected in mouse spleen and peripheral blood as well as in human marrow and peripheral blood.

The differentiation of erythroid cells in response to epo can also be studied in vitro by the incorporation of radioiron into hemoglobin. We have previously described a simplified method of measuring hemoglobin synthesis by this method in short-term rat marrow cultures. Hemoglobin synthesis in plasma clot cultures has been measured by the use of labeled leucine. In this study we used the cyclohexane-one extraction procedure to measure epo-induced hemoglobin synthesis associated with erythroid colonies and bursts by mouse marrow cells cultured in the methyl cellulose medium described by Iscove and Sieber.
MATERIALS AND METHODS

Preparation of Cell Suspensions
Bone marrow cells were flushed from the femora and tibiae of 12–16-wk-old female BD F1 mice into alpha medium (Flow Laboratories, Rockville, Md.). Cell clumps were dispersed by repeated flushing through a 22-gauge needle, and the cell suspension was filtered through a stainless-steel screen (100 mesh) before use. Cell counts were done on a hemocytometer, and all results are expressed as nucleated cells.

Colony Assays
The methyl cellulose culture medium of Iscove and Sieber was used to assay CFU-E and BFU-E. Mouse marrow cells were cultured at a density of 2 × 10^5 cells/ml in a mixture containing alpha medium, 0.8% methyl cellulose (4000 cps, Dow Chemical, Midland, Mich.), 30% fetal calf serum (Flow Laboratories), 1% bovine serum albumin (Armour Pharmaceutical, Chicago, Ill.), and 10^-4 M β-mercaptoethanol. All media contained gentamicin at 0.5 mg/ml (Schering, Kenilworth, N.J.). One-milliliter aliquots of the cell suspension were incubated in 35-mm-diameter dishes (model 5221-R, Lux Scientific, Thousand Oaks, Calif.) at 37°C in a fully humidified atmosphere of 5% CO2 in air. Four or five replicates were run for each experimental point, and the results are expressed as means ± 1 SD.

Erythroid colonies (8–64 cells) were counted after 2 days, and bursts (> 100 cells) were counted after 8 days. The colonies were routinely stained in situ with benzidine according to the method of Ogawa et al. and were counted at 25–40 X under a dissecting microscope. The determination of bursts was based on a combination of staining and morphology, since different degrees of staining are evident in bursts and since some nonerythroid colonies also exhibit benzidine-positive staining.

Hemoglobin Synthesis
For measurement of hemoglobin synthesis, marrow cells were cultured in the same manner as for the colony assays, with these exceptions: The cells were plated at higher densities, 5 × 10^6 to 1 × 10^7 cells/ml; the volumes of the cultures were decreased to 0.3 ml so that they could be cultured in the wells (16 mm diameter) of Costar tissue culture trays (model 3524, Costar, Cambridge, Mass.); six replicate cultures were run for each experimental point; the cells were incubated under a fully humidified atmosphere containing 3% CO2 in air, since the smaller-volume cultures tended to become more acidic under the higher CO2 atmosphere, causing decreased hemoglobin synthesis. The higher cell concentration was needed in order to get sufficient radioactivity in hemoglobin for accurate counting.

The cultures were labeled for 24 hr with 0.2 μCi (20 μl) of 59Fe-labeled rat transferrin prepared as follows: to 5.0 ml of frozen rat serum (previously heat-treated at 56°C for 1 hr) were added 4.0 ml of alpha medium, 0.5 ml of 0.9-M NaHCO3, and 0.5 ml of 59Fe (as 59FeCl3 in 0.1-M HCl, 200 μCi/ml, Amersham, Arlington Heights, Ill.); the thawed solution was mixed, incubated at 37°C for 30 min before use, and stored 4°C. In brief, the contents of each well were diluted with 1.0 ml of slightly hypertonic phosphate-buffered saline (PBS) and transferred to glass culture tubes (13 × 100 mm). The wells were washed with an additional 1.0 ml of PBS, and the tubes were centrifuged at 500–1000 g for 10 min at 4°C. The supernatant solutions were discarded, and the cellular macromolecular materials were precipitated by addition of 1.5 ml of ice-cold 5% trichloroacetic acid (TCA). The precipitates were washed once with 1.5 ml of the same solution and then were suspended in 0.5 ml of Drabkin’s solution (Acrule, Sigma Chemical, St. Louis, Mo.) and 0.1 ml of a carrier solution (2.6 mg/ml). Total uptake of 59Fe was determined at this point by counting the samples in an automatic gamma counter. Hematin was extracted by the addition of 0.1 ml of 1-M HCl and 2.0 ml of cyclohexanone (Eastman Kodak, Rochester, N.Y.). The samples were then mixed thoroughly and centrifuged at room temperature. It is important to do the extraction at room temperature because of the increased solubility of cyclohexanone in water at low temperatures. One-milliliter aliquots of the organic (upper) layer were removed for counting. The results are expressed as means ± 1 SD.

For measurement of hemoglobin synthesis associated with erythroid colony formation, radioiron was added on day 1, and the cultures were terminated 24 hr later. For measurement of hemoglobin synthesis associated with burst formation, one set of cultures was labeled 24 hr on day 5, one set was labeled on day 6, and one set was labeled on day 7. These cultures were terminated on days 6, 7, and 8, respectively. The mean hemoglobin synthesis from each set of cultures was totaled to give the value used for 6- to 8-day hemoglobin synthesis in the Results section.
Chloroform Extraction of Hematin

Cells labeled with $^{59}$Fe were centrifuged and washed two times with 2.0 ml of PBS, and hematin was extracted by the method of Fox and Thomson\(^5\) as follows: to each cell pellet, 2.0 ml of acetone were added, and, with constant mixing, 0.1 ml of 12-M HC1 was added drop by drop.

After centrifugation the supernatants were saved, and 1.0 ml of chloroform and 4.0 ml of a 1:1 mixture of 1-M HC1 and methyl alcohol were added. The chloroform (lower) layer was washed once with 5.0 ml of HC1:methanol (1:1) and then was counted on an automatic gamma counter.

Carboxymethyl Cellulose Chromatography of Hemoglobin

A modification of the method of Fantoni et al.\(^9\) was used for isolation of hemoglobin from the total cell extracts. Cells labeled with $^{59}$Fe were washed twice with PBS, and the cell pellet was resuspended in 1.0 ml of 0.1-M phosphate buffer, pH 6.5 (starting buffer). The cells were placed in an ice bath and broken by sonication for 30 sec at maximum power with a Kontes microultrasonic cell disrupter. The stroma was then removed by centrifugation. A 0.9-ml aliquot of the supernatant was layered on a carboxymethyl cellulose (CMC, Whatman CM-52, H. Reeve Angel Inc., Clifton, N.J.) column (0.5 × 5.0 cm). The column was washed with the starting buffer to elute the nonhemoglobin iron counts, and hemoglobin was eluted with 0.01-M phosphate buffer, pH 9.1. The elution of hemoglobin was monitored by measurement of the absorbance at 415 nm, and the $^{59}$Fe content of each fraction was determined. The remaining 0.1-ml aliquot was counted for total iron uptake, and the hematin iron was extracted by the cyclohexanone procedure described previously.

Erythropoietin

The erythropoietin used for these studies was a highly purified human urinary preparation dissolved in 0.1% bovine serum albumin in 0.15-M NaCl with 0.01-M CaCl\(_2\). The specific activity was 50,000 units/ml when determined in the starved rat in vivo assay;\(^21\) this represents a purity of about 60%.

The results presented in this report are representative of several different but consistent experiments done at different times.

RESULTS

We previously pointed out the advantages of using cyclohexanone\(^13\) in place of butanone-2\(^22\) for the extraction of hematin. We also reported that the cyclohexanone method appeared to extract appreciable amounts of nonheme radioactivity from long-term cultures, and we suggested that the chloroform extraction procedure of Fox and Thomson\(^18\) would be more specific for hematin iron from these long-term cultures. This matter was investigated further, and the findings are shown in Table 1. When TCA precipitation was used for preparation of the samples for extraction, instead of the original method of two washes with PBS and lysis of the cells with detergent, the cyclohexanone method was highly specific for hematin

<table>
<thead>
<tr>
<th>Epo (μ units/ml)</th>
<th>Treatment</th>
<th>Total iron Uptake</th>
<th>Extraction Method</th>
<th>Hematin</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>TCA ppt</td>
<td>131 ± 5</td>
<td>Cyclohexanone</td>
<td>40 ± 4</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>TCA ppt</td>
<td>206 ± 7</td>
<td>Cyclohexanone</td>
<td>48 ± 5</td>
<td>8</td>
</tr>
<tr>
<td>200</td>
<td>TCA ppt</td>
<td>237 ± 16</td>
<td>Cyclohexanone</td>
<td>54 ± 6</td>
<td>14</td>
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<tr>
<td>0</td>
<td>PBS wash</td>
<td>477 ± 14</td>
<td>Chloroform</td>
<td>39 ± 7</td>
<td>7</td>
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<tr>
<td>100</td>
<td>PBS wash</td>
<td>424 ± 13</td>
<td>Chloroform</td>
<td>48 ± 9</td>
<td>7</td>
</tr>
<tr>
<td>200</td>
<td>PBS wash</td>
<td>424 ± 120</td>
<td>Chloroform</td>
<td>55 ± 7</td>
<td>16</td>
</tr>
</tbody>
</table>

*Rat bone marrow cells were cultured in 0.2-ml cultures at 10\(^2\) cells/ml in 65% NCTC-109 containing 30-mM MOPS buffer at pH 6.8, 30% fetal calf serum, 5% rat serum, with 73 nmoles of unlabeled ferric nitrate per milliliter. The cells were incubated under 5% CO\(_2\) for 5 days and pulsed for 5 hr with 20 μl of $^{59}$Fe in 50% rat serum, 40% NCTC-109, 5% 0.9-M NaHCO\(_3\), and 5% $^{59}$Fe (200 μCi/ml).
radioactivity. The counts of the TCA-precipitated cyclohexanone-extracted hematin iron were virtually identical to the PBS-washed chloroform-extracted radioactivity. The difference between these results and those reported previously is probably due to the fact that the strong acid is much more effective than PBS in removing non-protein-bound inorganic iron, as can be seen in the results for total iron counts in Table 1. The PBS-washed control total iron counts were more than three times greater than those in the TCA control group. Additionally, no response was evident in the PBS-washed samples from cultures containing epo at 0.10 units/ml and 0.20 units/ml, whereas the TCA-precipitated samples at the same epo levels showed a response.

Table 2 lists the results of an experiment in which we measured hemoglobin synthesis by mouse marrow cells cultured in the presence and absence of 0.8% methyl cellulose. Stimulated hemoglobin synthesis by mouse cells in alpha medium without methyl cellulose was about the same at day 2 as that by cells cultured in alpha medium with methyl cellulose. By day 3, stimulated hemoglobin synthesis in the cultures with methyl cellulose was still at a relatively high level, 50% of that at day 2; however, in cultures without methyl cellulose, hemoglobin synthesis fell nearly to zero at day 3. The rate of stimulated hemoglobin synthesis at day 7 in cultures of mouse marrow containing methyl cellulose was almost four times as great as the rate of synthesis at day 2, showing that there was a second very large wave of hemoglobin formation in these cultures. Semisolid medium seems to be a requirement for long-term hemoglobin synthesis, since the rate at day 7 in mouse marrow cultures without methyl cellulose was only 2% of the rate of synthesis in mouse marrow cultures with methyl cellulose.

The possibility that the use of methyl cellulose in the culture system might interfere with the cyclohexanone extraction of hematin was investigated by thin-layer chromatography. The results (Table 3) show that the method is very specific for hematin iron under these conditions. Two major iron-porphyrin components, with Rf values of 0.55 and 0.65, were resolved by the thin-layer system, as had been shown previously for short-term rat marrow cultures. In the experiment shown, 5% of the counts remained at the origin, possibly representing irreversible adsorption of hematin to the silica gel, since a small amount of color remained at the origin as well.

In short-term marrow cultures, essentially all of the hematin radioactivity is derived from hemoglobin. The relationship between hematin radioactivity and hemoglobin in 7-day mouse marrow cultures was studied by carboxymethyl cellulose chromatography. The fractions containing the majority of hemoglobin eluted from the column had a constant ratio of iron radioactivity to absorbance at

<table>
<thead>
<tr>
<th>Table 2. Effect of Methyl Cellulose on Hemoglobin Synthesis by Mouse Marrow Cells*</th>
</tr>
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<tbody>
<tr>
<td>Medium</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Epo</td>
</tr>
<tr>
<td>With methyl cellulose</td>
</tr>
<tr>
<td>Without methyl cellulose</td>
</tr>
</tbody>
</table>

*Mouse bone marrow cells were incubated at 10⁶ cells/ml in medium containing 0.8% methyl cellulose as described in the text or in the same medium without methyl cellulose. Stimulated cultures were given epo at 5 units/ml.
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Table 3. Thin-Layer Chromatography of Cyclohexanone-Extracted Hematin*

<table>
<thead>
<tr>
<th>Peak Rf</th>
<th>Fe in Peak (cpm)</th>
<th>Percentage of Total cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>188</td>
<td>5</td>
</tr>
<tr>
<td>0.55</td>
<td>412</td>
<td>11</td>
</tr>
<tr>
<td>0.65</td>
<td>3150</td>
<td>84</td>
</tr>
</tbody>
</table>

*Extracts of six replicate cultures (8 days) were pooled, concentrated to dryness, dissolved in a small volume of chloroform:methanol:pyridine (10:25:3) (vol:vol:vol) and applied to a silica gel thin-layer foil. The chromatogram was developed immediately with the same solvent, dried, and scanned on a radiochromatogram scanner.

415 nm and represented 26.2% of the total iron counts. This result was in very close agreement with the results of cyclohexanone extraction from a separate aliquot, which yielded 25.6% of the total counts as hematin iron. Furthermore, when the column eluates were extracted with cyclohexanone, the nonhemoglobin fractions yielded only 1% of their total counts into the cyclohexanone layer, showing that the cyclohexanone procedure does not extract nonhemoglobin counts.

Work in this laboratory has shown that rat marrow cells utilize iron from rat transferrin to a much greater extent than from transferrin of other species.23 Because other laboratories have reported the use of iron citrate, with no additional source of transferrin, to measure hemoglobin synthesis in cultures of mouse liver cells,24 the transferrin requirements of long-term mouse marrow cultures for maximum iron incorporation into hemoglobin were investigated. The use of rat serum containing labeled iron resulted in 5.7-fold greater incorporation than similarly prepared iron in mouse serum (Table 4, experiment 1) and 6-fold greater incorporation than iron added as iron citrate (Table 4, experiment 2). Both sera were about the same degree of saturation.

The time courses of total iron uptake and of hemoglobin synthesis by mouse bone marrow cells cultured in methyl cellulose are shown in Fig. 1.Total iron uptake by nonstimulated cells fell after 1 day and then gradually rose through day 10 (Fig. 1A). This must be due to the incorporation of iron into TCA-precipitable material other than hemoglobin since the time course of hemoglobin synthesis by the same cells (Fig. 1B) dropped rapidly to zero by the third day and remained at zero through the 10th day. In addition, no hemoglobin-containing colonies were formed in these cultures in the absence of epo.

In the presence of epo, the level of total iron uptake by the cells was the same on

Table 4. Effects of Different Transferrins on Hemoglobin Synthesis by Mouse Marrow Cells*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of Transferrin</th>
<th>Total Added Radioactivity (cpm)</th>
<th>Hemoglobin (cpm)</th>
<th>Percentage Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rat</td>
<td>53,300</td>
<td>237 ± 68</td>
<td>0.44 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>69,700</td>
<td>53 ± 39</td>
<td>0.07 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>Rat</td>
<td>48,000</td>
<td>576 ± 70</td>
<td>1.20 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>55,800</td>
<td>123 ± 38</td>
<td>0.22 ± 0.07</td>
</tr>
</tbody>
</table>

*Mouse marrow cells were incubated at 5 × 10⁶/ml in experiment 1 and 10⁷/ml in experiment 2. The serum-free ⁶⁷Fe in experiment 2 contained 0.01-M citrate in alpha medium. In both experiments the incubation period was 7 days and the cultures contained epo at 5 U/ml.
days 1 and 2. It then increased gradually through day 4, after which it rose rapidly to a peak on day 6 with epo at 0.5 unit/ml and on day 7 with 5 unit/ml. By day 10, total iron incorporation was at control levels with both doses of epo.

Epo-stimulated hemoglobin synthesis was increased at day 2, as had previously been demonstrated, corresponding to the time when the number of erythroid colonies was at a maximum. A second and much larger effect occurred at day 7; it decreased gradually through day 9 and was at control levels on day 10. The time course of hemoglobin synthesis at the later times corresponds to the time course of benzidine-positive bursts, which are maximum at day 8 and decrease thereafter (Fig. 2). The shoulder at day 5 in the curve for stimulated hemoglobin synthesis at an epo concentration of 5 unit/ml (Fig. 1B) is reproducible and may represent hemoglobin synthesis due to the early day-3 bursts described by Gregory,25 which appeared on days 3–4 and disappeared by days 5–6.

One criterion for a parameter to be a measure of hormone action on target cells is the existence of a dose–response relationship. The curve of epo dose versus CFU-E response shown in Fig. 3A is similar to those reported by other laboratories8,25 and has a plateau above 0.3 unit/ml. The curve of epo dose versus hemoglobin synthesis response also showed a semilogarithmic relationship, with a plateau at about 0.6 unit/ml in the experiment shown. There was no significant decrease in erythroid colonies or in day-2 hemoglobin synthesis at doses of epo as high as 10 unit/ml. Since the epo used in this experiment was highly purified, the result indicates that extremely high doses of epo are not toxic to the cells.

Since the peak of stimulated hemoglobin synthesis related to burst formation is quite broad, beginning at day 5 and continuing until day 10 (Fig. 1B), a single
Fig. 2. Time course of benzidine-positive bursts. Mouse marrow cells were incubated at 2 \( \times 10^4 \) cells/ml with Epo at 2 units/ml. Each point represents the mean counts of five replicate plates on the day indicated.

24-hr labeling period will not be representative of the entire peak. Therefore, the 6-, 7-, and 8-day time points were chosen, from a number of different time studies, as being representative of the peak. These times are also late enough to ensure no contribution from hemoglobin synthesis associated with the progeny of CFU-E. We set out to determine if we could measure 6–8-day hemoglobin synthesis with one 72-hr labeling period in one set of cultures instead of using three 24-hr labeling periods in three sets of cultures. The single 72-hr labeling period gave, on the
good agreement with the slopes reported by other laboratories for CFU-E. The average, only 64% as much iron incorporation into hemoglobin as the sum of the iron incorporation into hemoglobin from the three shorter labeling periods (data not shown). We therefore used the sum of the hemoglobin measurements from three sets of 24-hr labeled cultures to estimate burst-associated hemoglobin synthesis.

The validity of this estimation of hemoglobin synthesis related to burst formation can be seen in the curve of the log epo dose versus hemoglobin synthesis shown in Fig. 3B. There was a linear response between epo concentrations of 0.25 and 10 unit/ml. The relationship for burst number is shown for comparison. The linear portion of the semilogarithmic curve was between epo concentrations of 0.2 and 3 unit/ml. The plateau epo dose was between 3 and 5 unit/ml when the burst number was measured, whereas no plateau was seen in hemoglobin synthesis at epo doses as high as 10 unit/ml. The difference between the slopes for hemoglobin synthesis and burst formation above 2.5 unit/ml is significant at the \( p < 0.001 \) level.

Another criterion of a successful assay for hormone action on target cells is evidence of a relationship between cell number and response at a given hormone level. The effects of cell number on CFU-E and day-2 hemoglobin synthesis are compared in Fig. 4. Both parameters had an excellent fit when plotted as log parameter versus log cell number. The curves for CFU-E and hemoglobin synthesis are essentially parallel, with slopes of 1.2 and 1.1, respectively. The values are in

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**Fig. 4.** Effect of cell number on hemoglobin synthesis and colony number at early and late times. Closed circles represent hemoglobin synthesis; open circles represent colony counts. A: day-2 hemoglobin synthesis in cultures containing epo at 5 units/ml and CFU-E counts of four cultures containing the same dose of epo; B: 6-8-day hemoglobin synthesis in cultures containing epo at 20 units/ml and BFU-E counts in four cultures containing the same dose of epo.
parallelism of the two lines indicates that hemoglobin synthesis at day-2 can be used as a measure of CFU-E.

The relationship between cell number and response at days 6–8 is shown in Fig. 4. The range of cell concentrations at which bursts can be studied is much more limited than is the case for 2-day colonies, since the number of bursts is greatly reduced at concentrations above $10^6$ cells/ml. Both 6–8-day hemoglobin synthesis and erythroid bursts showed good linearity for the plots of log cell number versus log response. The curves of log cell number versus log response for the two parameters were not parallel, however, as they were for the day-2 cultures. The slope for the burst line was 1.2, and over a series of experiments the range was 1.1–1.4. This is in fair agreement with the slope 1.1 reported by Iscove and Sieber. The slope for the 6–8-day hemoglobin synthesis relationship, however, was 1.8 and was consistently higher, with a mean value of 1.7 and a range of 1.5–2.0 for eight experiments. This means that the 6–8-day parameters cannot be substituted for each other without correction for the difference in slopes.

**DISCUSSION**

The cyclohexanone method is a fast and simple procedure for extraction of hemoglobin iron from cultured bone marrow cells. We showed that it is specific for hematin radioactivity, since samples from 5-day cultures of rat marrow cells prepared by acid precipitation yielded the same number of counts into cyclohexanone as were extracted with chloroform from PBS-washed samples. Additionally, when cyclohexanone extracts of 7-day mouse marrow cultures were examined by thin-layer chromatography, 95% of the radioactivity migrated with hematin. The fact that the percentage of radioactivity eluted from a carboxymethyl cellulose column in the fractions containing hemoglobin was virtually the same as that extracted into cyclohexanone from an aliquot of the same sample indicates that hemoglobin is the predominant heme protein in these cultures.

There appears to be a requirement for some kind of support in the medium so that long-term epo-induced hemoglobin synthesis will be elicited in these cultures. The semisolid medium may provide the cells with a generalized three-dimensional environment that might be required for growth and differentiation of erythroid cells, or it may simply prevent the cells from attaching to the dish, which might prevent differentiation. Dexter and Lajtha have had success in maintaining hemopoietic stem cells and granuloid precursors for long periods of time by culturing mouse marrow cells in liquid medium on top of established monolayers of cocultured thymus and marrow cells or monolayers of marrow cells alone. The existing monolayer in their cultures may prevent further attachment of the suspended cells, as well as having more specific conditioned medium or microenvironmental effects.

The low proliferative capacity and early time of response of CFU-E indicate that they are late erythroid progenitors, i.e., they are quite near the mature cells in the differentiation pathway. The high proliferative capacity and late time response of the BFU-E indicate that they are early erythroid progenitors, quite near the hemopoietic stem cell in the differentiation pathway. The so-called day-3 BFU-E reported by Gregory to produce small bursts consisting of four or more small erythroid colonies, first appearing at day 3 and disappearing by day 6, would, by
the preceding criteria, be an intermediate burst-forming unit. The shoulder appearing at day 5 in the time course of epo-induced hemoglobin synthesis (Fig. 1), which is reproducible, may represent these intermediate forms.

Erythroid colony counts and day-2 hemoglobin synthesis gave similar results in experiments measuring the response to graded doses of epo and to increasing cell concentration, suggesting that the two parameters can be substituted directly for each other. The results of measurements of 6–8-day hemoglobin synthesis differ from those for burst numbers in both types of experiments.

In the epo dose–response experiments at 6–8 days, the curve for colony counts reached a plateau at 3–4 unit/ml, whereas no plateau was evident in the curve for hemoglobin synthesis even at 10 unit/ml. This difference means that at high levels epo causes an increase in the rate of hemoglobin synthesis in the individual bursts, since hemoglobin synthesis increases with a constant number of bursts. A possible explanation for this result may be that these levels of epo cause increased amplification of the BFU-E progeny, resulting in increased numbers of hemoglobin-synthesizing cells in the bursts. Epo doses above the plateau level seem to result in larger bursts. However, the size of these bursts is difficult to quantitate because of the large variation in the size and compactness of the bursts in any single plate.

We have shown that hemoglobin synthesis can be used as a probe for study of the differentiation of early erythroid progenitors, the BFU-E, which previously have been investigated only in clonal assays. Measurement of hemoglobin synthesis can provide additional information about the differentiation of the cells in the burst, whereas burst counts provide information only about the number of progenitor cells.

REFERENCES

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