Hemoglobin Synthesis in Human BFU-E and CFU-E-Derived Erythroid Colonies

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We have analyzed the synthesis of fetal hemoglobin (HbF) in the differentiated erythroid colonies produced by erythropoietin-responsive committed erythroid progenitors in plasma cultures of human marrow and peripheral blood. Two independent biochemical techniques, carboxymethyl-cellulose column chromatography of 3H-leucine-labeled globin, and polyacrylamide gel electrophoresis of 59Fe-labeled hemoglobin were used to quantitate the hemoglobin(s) synthesized in replicate cultures containing varying concentrations of the erythropoiesis-stimulating hormone erythropoietin. Excellent quantitative agreement between these two methods was observed. Sensitivity of these assay methods was such that 5% fetal hemoglobin could be detected. In separate studies of three normal adult males, we observed little or no detectable fetal hemoglobin synthesis in the differentiated colonies derived from marrow erythroid progenitors over a range of erythropoietin concentration that produced increasing numbers and size of erythroid colonies. Peripheral blood erythroid progenitors of these same individuals were studied in cultures containing 0.5 U/ml and 2.0 U/ml of erythropoietin. At both these erythropoietin doses, fetal hemoglobin was synthesized at concentrations varying between 9% and 39% of the adult hemoglobin. Cytoplasmic RNA was isolated from the differentiated progeny of peripheral blood erythroid progenitors to permit analyses using radioactive cDNA probes specific for human α-, β-, and γ-globin mRNA. Results from the RNA-excess cDNA liquid hybridization experiments also indicated that γ-globin mRNA was present in peripheral blood BFU-E-derived erythroid colonies at a level comparable to the amount of γ-globin chain synthesis observed. In contrast, erythroid colonies derived from marrow and blood erythroid progenitors of three hematologically abnormal individuals synthesized approximately equal amounts of fetal hemoglobin in plasma culture. Once again, the level of HbF synthesis was unrelated to erythropoietin concentration in culture. We conclude that the erythroid progenitor cells ordinarily found in the peripheral blood (F progenitors) are programmed to produce differentiated erythroid colonies that synthesize fetal hemoglobin. The great majority of normal bone marrow erythroid progenitors are not programmed to produce colonies that synthesize hemoglobin F, but under conditions of anemic stress, the population of F progenitors within the marrow expands sufficiently to populate erythroid cultures with colonies that produce considerable quantities of hemoglobin F.
IN THE 8-wk-old human fetus, more than 90% of the hemoglobin present in peripheral blood erythrocytes is of the fetal variety (HbF: α2γ2). By 200 days postpartum, however, the level of HbF in the blood is less than 1% of that of adult hemoglobin (HbA: α2β2), and circulating cells that contain any HbF are a rarity. The switching of bone marrow cells from HbF to HbA synthesis is not normally reversed during adult life. However, a number of exceptions to this rule are known. Approximately 20% of women synthesize and accumulate up to 3.5% HbF during the second trimester of pregnancy. More importantly, a number of common nonmalignant dysfunctions of erythropoiesis, including sickle cell anemia, β-thalassemia, and aplastic anemia, are associated with an elevated level of HbF synthesis. In isolated instances, such as the Shi Arab population homozygous for sickle cell anemia, raised HbF levels in the peripheral blood can fully alleviate what would otherwise be a potentially lethal hemoglobinopathy. Thus, understanding the control mechanism involved in HbF production in human adults may have great practical importance.

A number of animal models have been studied in an effort to examine the mechanisms that control the switch from the synthesis of one globin chain type to another. Perhaps the best characterized system is the switch from βA- to βC-globin synthesis, which accompanies anemia in sheep and goats. The results of these studies strongly implicate the hormone erythropoietin in the control of this switching process. Erythroid colonies derived from the marrow progenitors of sheep and goats synthesize higher levels of HbC in culture in response to increasing doses of erythropoietin. It has recently been reported that colonies derived from cultured human erythroid progenitor cells exhibit a dose response to erythropoietin with respect to HbF synthesis. Papayannopoulou et al. have also reported marked stimulation of fetal hemoglobin synthesis in cultured human erythroid colonies derived from bone marrow progenitors.

In this study, we have used the plasma culture system originally described by Stephenson et al. to perform biochemical analyses of the proteins and RNA synthesized by the erythroid progeny of erythropoietin-responsive erythroid progenitor cells from both human marrow and peripheral blood. This utilized the CMC-column chromatography procedure, and an excellent separation of γ-globin and nonglobin protein has been achieved. As an alternative independent method to evaluate levels of fetal hemoglobin synthesis, polyacrylamide slab gel electrophoresis of 59Fe-labeled hemoglobin was performed. Our results contradict previously reported data in that fetal hemoglobin synthesis usually constituted less than 5% of adult hemoglobin synthesis in normal bone marrow cultures at all concentrations of erythropoietin tested. In contrast, a high level of fetal synthesis was observed in erythroid cell cultures of normal blood and in both marrow and blood erythroid cell cultures from hematologically abnormal individuals. A preliminary report of this work has been published elsewhere.

MATERIALS AND METHODS

Cell Samples

Posterior iliac marrow aspirates and peripheral blood samples were obtained with informed consent from three normal males, from two patients with sickle cell anemia (SCA) who had circulating HbF/HbA + HbF of 8%–15%, and from one patient with transient erythroblastopenia of childhood...
Marrow samples were obtained on two occasions from one of the patients with SCA. Great care was exercised to avoid excessive contamination of the marrow cells with peripheral blood, and marrow aspirates were always 0.5 ml or less. Estimates based on total cell count indicated that peripheral blood contamination of the marrow samples was less than 10%. Peripheral blood samples of 50–100 ml were drawn by standard venipuncture techniques. Both marrow and peripheral blood cells were adjusted to 0.4% sodium citrate to prevent coagulation. Mononuclear cells were separated from erythrocytes by Ficoll-Hyphaque density centrifugation as previously described. The patient with TEC was a 2-yr-old child who had been entirely well until 4 wk prior to admission when pallor was noted. On admission, her hemoglobin was 5 g/100 ml. The reticulocyte count was less than 1% and the mean corpuscular volume 82 cuu. No fetal hemoglobin was detected in her blood either by electrophoresis or by the acid elution technique. The marrow aspirate revealed an M:E ratio of 6:1. Nearly all of the recognizable erythroid cells were proerythroblasts. No treatment was given other than a transfusion of packed red cells. Five days after the marrow aspirate, increased reticulocytes were noted in the patient’s blood. One week later, fetal hemoglobin was increased to 3.5% by electrophoresis and alkali denaturation, and as many as 10% fetal hemoglobin-containing cells were seen in the blood smear. The patient has gone on to complete recovery. Fetal hemoglobin is now less than 1% of the total circulating hemoglobin concentration.

**Cell Culture**

Marrow and peripheral blood mononuclear cells were plated at 5 x 10^3–10^6 nucleated cells/ml and 5 x 10^6 nucleated cells/ml, respectively, in the plasma clot culture system, as previously described. Human urinary erythropoietin (lot ARG-8-Ta-LSL, kindly provided by Dr. Anne Ball, N.I.H.) was added at the desired concentration at zero time. Cultures were incubated in an atmosphere of high humidity and 95% air/5% CO2 at 37°C for either 5 days (CFU-E-derived colonies) or 11 days (BFU-E-derived colonies). CFU-E-derived colonies were pulse labeled for 6 hr on day 5 by overlaying 50 μCi of [3H]-leucine (New England Nuclear, 40–60Ci/mMole) in 100μl of NCTC-109 onto 1 ml of plasma clot. The identical protocol was used to isotopically label BFU-E-derived colonies on day 11. Four milliliters of plasma clot were generally used for each erythropoietin concentration. CFU-E- and BFU-E-derived erythroid colonies were similarly isotopically labeled for a 24-hr period on the above days using 50μCi of 59FeCl2 (New England Nuclear, 20–40 Ci/g iron) in 100μl of fetal calf serum. Two milliliters of plasma clot were labeled with 59Fe at each erythropoietin concentration.

**Globin Analysis**

CMC-column chromatography of the 3H-leucine-labeled proteins synthesized in plasma culture was performed essentially as previously described. Briefly, after incubation with isotope, the plasma clots were washed twice in 3 vol of normal saline by centrifugation at 1500 rpm for 2 min at 4°C. The washed clots were resuspended to a final volume of 0.5 ml in distilled water, and cells were disrupted by two cycles of freeze-thawing at -70°C. Approximately 15 mg of both cord blood hemolysate and adult blood hemolysate was added to each sample to act as a combined carrier and internal standard before globin was prepared by the cold acid acetone technique. Samples were stored at -70°C until analyzed. Globins were eluted from the CMC column using a nonlinear phosphate gradient with a starting buffer of 3.5 mM sodium phosphate, pH 6.6, and finishing buffer of 35 mM sodium phosphate, pH 6.7. Sixty-five fractions of 7 ml each were collected from the column, and 3-ml aliquots were counted in 10 ml of Instagel using a Packard liquid scintillation counter. The γ/α and β/α ratios were determined by integrating the total number of counts minus background under each peak, as previously described.

**Hemoglobin Analysis**

Native polyacrylamide slab gel electrophoresis (PAGE) of 59Fe-labeled hemoglobin was performed essentially as described. In this assay, plasma clots were disrupted by digestion with pronase, and the liberated cells were collected by centrifugation at 1500 rpm for 5 min at 4°C. The cell pellet was washed once in phosphate-buffered saline (PBS) minus calcium and magnesium, followed by centrifugation at 1500 rpm for 5 min at 4°C. The cell pellet was then lysed in 30 μl of cold distilled water for 1 min followed by centrifugation at 2000 rpm for 5 min at 4°C. Ten-microliter aliquots of the cell-free supernatant were then stored at -70°C until assayed by PAGE. All samples were electrophoresed without added hemoglobin carrier to avoid possible heme–heme exchange. Gels were then dried under
vacuum with heat, and autoradiograms were made using Kodak NS-2T film. The relative percentage of 59Fe-labeled HbA and HbF was quantitated by cutting out that portion of the original gel that both corresponded to a band on the autoradiogram and comigrated with authentic nonradioactive cord blood hemoglobin. The cut-out portions of the gel were then counted in a Beckman gamma counter.

**Messenger RNA Analysis**

RNA-cDNA hybridization was performed as previously described. Cytoplasmic RNA was prepared from cells released from 12-day plasma cultures by pronase digestion, and the liberated cells were collected by centrifugation at 1500 rpm for 5 min at 4°C, followed by 1 wash in cold PBS minus calcium and magnesium, and recentrifuged at 1500 rpm for 5 min at 4°C. The cell pellet was resuspended in 0.5 ml of cold Triton-X-100 lysis buffer (0.15 M NaCl; 50 mM Tris-HCl, pH 8.5; 1.5 mM MgCl2; 0.1% TX-100; 0.15% diethylpyrocarbonate) and vortexed for 1 min. Nuclei were pelleted at 1500 rpm for 3 min at 4°C, and 0.5 ml of 2X SDS buffer (0.1 M NaCl; 10 mM Tris-HCl, pH 7.3; 1 mM EDTA; 1% SDS) was immediately added to the supernatant fluid. Total cytoplasmic RNA was then phenol-extracted in the presence of 100 µg of *Escherichia coli* carrier tRNA. RNA was ethanol precipitated at −20°C by the addition of 3 vol of 100% ethyl alcohol to 1 vol of RNA solution, previously adjusted to a concentration of 0.1 M sodium acetate, pH 4.5. The RNA pellet was routinely resuspended in 60 µl of deionized water. The synthesis and purification of cDNA probes specific for human α-, β-, and γ-globin mRNA have been described elsewhere.

**RESULTS**

We have approached the problem of fetal hemoglobin synthesis in adult cells by simultaneous culture of marrow and peripheral blood erythroid progenitor cells from each individual under identical culture conditions. In each experiment, the erythropoietin dose was varied systematically to analyze the potential effects of erythropoietin on the ratio of HbA/HbF synthesis. The growth characteristics of marrow and peripheral blood erythroid progenitors were significantly different. In marrow cultures, colonies of the CFU-E type, i.e., single, compact, erythroid clones of 4–64 cells, were observed after 5–7 days of incubation. These colonies matured and then lysed by 10–12 days of incubation. Between 10 and 14 days, a second population of colonies of the BFU-E type, i.e., multicentric erythroid colonies containing 64–10,000 cells, were observed. Biochemical studies on marrow cultures were therefore performed on replicate sets of cultures with one group of cultures studied between 5 and 7 days of incubation and a second set between 10 and 12 days of incubation. Since only BFU-E are found in significant numbers in peripheral blood, biochemical analysis of these cultures was performed between days 10 and 12. In marrow, significant numbers of CFU-E- and BFU-E-derived colonies were observed at an erythropoietin dose of 0.1 U/ml. As previously noted, peripheral blood BFU-E require a higher concentration of erythropoietin for maximal colony development.

**Synthesis of Globin in Normal Bone Marrow CFU-E Cultures at Varying Concentrations of Erythropoietin**

Figure 1 is representative of the results obtained after culture of normal human bone marrow from a single individual (D.H.) at various concentrations of erythropoietin. All cell cultures were done in parallel with the same tissue culture reagents. CFU-E marrow cultures were pulsed with 3H-leucine for 6 hr on day 5. 3H-globin was prepared, and CMC chromatography was performed as described in Materials and Methods. In CFU-E marrow cultures without added erythropoietin, 3H-leucine incorporation was consistently observed in the pre-γ, mid-β-α, pre-α, and α regions.
Fig. 1. CMC-column chromatography of 3H-leucine-labeled proteins synthesized on days 5–6 in plasma cultures containing bone marrow cells from one individual (D.H.) and varying concentrations (IU/ml plasma culture) of human erythropoietin. Human α-, β-, and γ-globin prepared from cord blood hemolysates (-----) were used as carrier and internal standard for radioactive globins (++++) synthesized in culture.
Table 1. Quantitation of Fetal Globin and Hemoglobin Synthesis in Human Marrow and Peripheral Blood Cell Cultures

<table>
<thead>
<tr>
<th>Erythropoietin (U/ml)</th>
<th>Marrow CFU-E</th>
<th>Marrow BFU-E</th>
<th>Blood BFU-E</th>
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<td>0.1</td>
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Normal individuals

Experiment D.H.
- Globin CMC chromatography: <0.05, <0.05, <0.05, <0.05, <0.05, <0.05, 0.15, 0.16
- Hemoglobin PAGE: <0.02, <0.02, <0.02, <0.02, <0.02, <0.02, 0.16, 0.19

Experiment B.C.
- Globin CMC chromatography: <0.05, <0.05, <0.05, <0.05, <0.05, <0.05, 0.33, 0.39
- Hemoglobin PAGE: <0.02, <0.02, <0.02, <0.02, <0.02, <0.02, 0.24, 0.32

Experiment D.N.
- Globin CMC chromatography: <0.05, <0.05, <0.05, <0.05, <0.05, <0.05, 0.09, 0.10
- Hemoglobin PAGE: <0.02, <0.02, <0.02, <0.02, <0.02, <0.02, 0.06, 0.09

Hematologically abnormal individuals

Transient erythroblastopenia
- Globin CMC chromatography: <0.05, <0.05, <0.05, <0.05, <0.05, <0.05, 0.19, 0.14
- Hemoglobin PAGE: <0.02, <0.02, <0.02, <0.02, <0.02, <0.02, 0.39, 0.37

Sickle cell anemia—study 1
- Globin CMC chromatography: 0.42, 0.47, 0.44, 0.40, 0.37, 0.40, 0.49, 0.50
- Hemoglobin PAGE: 0.39, 0.41, 0.42, 0.47

Sickle cell anemia—study 2
- Globin CMC chromatography: 0.35, 0.39, 0.44, 0.40, 0.41, 0.42, 0.47
- Hemoglobin PAGE: <0.02, <0.02, <0.02, <0.02, <0.02, <0.02, 0.06, 0.09

Data represent single determinations of globin and hemoglobin.

*Incubation of freshly explanted SCA marrow and blood erythroid precursors with 3H-leucine yielded γ/α ratios of 0.035 and 0.049, respectively.

of the column (Fig. 1). A modification of the salt gradient used for elution (see Materials and Methods) in the standard CMC-column technique markedly improved the separation of the pre-γ and γ peaks. When 3H-globin was prepared from CFU-E marrow cultures containing erythropoietin, both α- and β-globin were synthesized (Fig. 1) as judged by comigration with authentic α and β globin. The β/α ratios observed for CFU-E marrow cultures that contained 0.1, 0.5, and 2.0 IU/ml erythropoietin were 0.73, 0.72, and 0.69, respectively. Similar results were obtained with CFU-E marrow cultures from two other individuals (Table 1). No significant synthesis of γ-globin was observed at any concentration of erythropoietin.

Synthesis of Globin in Normal Bone Marrow BFU-E Cultures at Varying Concentrations of Erythropoietin

Experiments identical to those above were performed with the same marrow samples on day 11. At this time, marrow BFU-E-derived colonies are at or near maximal numbers, while most CFU-E-derived colonies have lysed. The pattern of globin synthesis observed in marrow BFU-E cultures was essentially identical to CFU-E marrow cultures. Substantial synthesis of α- and β-globin was observed at all concentrations of erythropoietin tested (Fig. 2). No significant synthesis of γ-globin was observed in any of the cultures (Fig. 2, Table 1). Synthetic ratios of
β/α in the BFU-E marrow cultures containing 0.1, 0.5, and 2.0 IU/ml erythropoietin were 0.80, 0.86, and 0.90, respectively.

Synthesis of Globin and Globin mRNA in Peripheral Blood BFU-E Cultures

Blood from both normal individuals and those with sickle cell anemia contains significant numbers of BFU-E but virtually no CFU-E. Blood BFU-E cultures

Fig. 2. CMC-column chromatography of ³H-leucine-labeled proteins synthesized on days 11–12 in plasma cultures containing bone marrow cells from one individual (D.H.) and varying concentrations of erythropoietin.
containing 2.0 IU/ml erythropoietin were therefore labeled with $^3$H-leucine on day 11 only. Globin was prepared and processed as described above. It is immediately evident from Fig. 3 that a substantial amount of $\gamma$-globin is synthesized in BFU-E-derived erythroid colonies of both normal individuals and those with sickle cell anemia (Table I).

The synthesis of $\gamma$-globin by blood BFU-E from normal individuals has been confirmed by mRNA-cDNA hybridization (Fig. 4). In this companion experiment, total cytoplasmic RNA was extracted from $2 \times 10^7$ cells harvested on day 12 from 10 ml of peripheral blood BFU-E cultures containing 2.0 IU/ml erythropoietin. RNA purification and RNA-DNA liquid hybridization were then performed. Sufficient globin mRNA was present in this preparation to easily saturate radioactive cDNA probes specific for $\alpha$- and $\beta$-globin mRNA. There was also significant hybridization to a cDNA probe for $\gamma$-globin mRNA. In this experiment (individual B.C.), $\gamma$-globin mRNA represented about 20% of the total globin mRNA, in rough agreement with Figs. 3 and 5 and Table 1. Our experience to date indicates that the
cells of blood BFU-E-derived erythroid colonies synthesize substantially greater amounts of γ-globin mRNA than do the cells of marrow BFU-E-derived erythroid colonies. At present, however, technical difficulties in preparing cDNA, which is absolutely specific for γ-globin mRNA, has severely restricted these studies. Hopefully, use of the human gene clones for α-, β-, and γ-globin will alleviate this problem.

Synthesis of Fetal Hemoglobin in Bone Marrow and Peripheral Blood Cell Cultures at Varying Concentrations of Erythropoietin

To facilitate the rapid screening of multiple human marrow and peripheral blood cell cultures for their hemoglobin synthesis, we labeled the heme moiety of hemoglobin with 59Fe and analyzed the radioactive hemoglobin(s) by native polycrylamide slab gels. In this experiment, marrow CFU-E and BFU-E, as well as peripheral blood BFU-E from one individual (B.C.) were simultaneously grown in plasma cultures containing varying concentrations of erythropoietin. Cultures were pulsed with 59Fe, as described in Materials and Methods. The radioactive iron-containing proteins synthesized were then analyzed by native PAGE, and an autoradiogram of the dried gel was prepared. From Fig. 5, it is evident that a prominent iron-containing protein is synthesized in all cell cultures, i.e., its synthesis is erythropoietin-independent. Since this band comigrates with an authentic bovine ferritin standard located by staining with ferrocyanide,10 we believe it to

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Fig. 4. RNA-excess liquid hybridization to tritiated cDNA specific for human α (O----O), β (■—■), and γ (▲—▲) globin mRNA. Total cytoplasmic RNA was prepared from peripheral blood BFU-E-derived colonies after incubation in plasma cultures containing 2.0 IU/ml of human erythropoietin for 12 days. Peripheral blood cells were from one individual (B.C.).

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Fig. 5. Autoradiogram of a polycrylamide slab gel after electrophoresis of 59Fe-labeled proteins synthesized in plasma cultures containing either bone marrow or peripheral blood mononuclear cells from one individual (B.C.) and varying concentrations of human erythropoietin (Epo). CFU-E and BFU-E cultures were pulsed with 59Fe for 24 hr on days 5–6 and 11–12, respectively.
be ferritin. In both marrow CFU-E and BFU-E cultures at all erythropoietin concentrations, a single Fe-containing protein that comigrates with authentic human HbA is present. No such protein is synthesized in cultures without added erythropoietin. Peripheral blood BFU-E cultures from this individual synthesized two iron-containing proteins in addition to ferritin. These bands comigrated with authentic human HbA and HbF. In most of the marrow and peripheral blood samples, a faint band located approximately midway between HbA and ferritin, was observed. Since this band comigrates with purified human HbA2 and routinely was present at 1%-2% of the concentration of HbA, we believe it to be HbA2. The presence of this band emphasizes the sensitivity of the PAGE technique.

In Table 1, we have summarized the results of our studies of globin and hemoglobin synthesis in replicate cell cultures of marrow and peripheral blood from three normal individuals, two patients with sickle cell anemia, and one patient with TEC. Qualitative and quantitative agreement between the standard method of analysis, CMC-column chromatography of globin polypeptide chains, and gel electrophoresis of 59Fe-labeled hemoglobin is excellent. Both techniques of analysis independently indicate that normal marrow CFU-E and BFU-E cultures at low or high erythropoietin concentrations synthesize more than 98% HbA. In contrast, peripheral blood BFU-E cultures from the same individuals always synthesized HbF at quite high levels. This result underscores the requirement in such studies for marrow samples uncontaminated by peripheral blood cells. Although undetectable or very low levels of HbF synthesis were observed in normal marrow cultures, we confirm the original observation9 that 30%-40% of the hemoglobin synthesized in both BFU-E and CFU-E marrow cultures from individuals with sickle cell anemia is HbF. Marrow BFU-E and CFU-E cultures from one patient with TEC known not to have significant numbers of F cells in the peripheral blood at the time of marrow aspiration also synthesized HbF in culture. It is noteworthy that within 2 wk of marrow sampling, the peripheral erythrocytes of this individual contained 3.5% HbF (see Materials and Methods). Thus, we were able to detect HbF synthesis in plasma cultures of human marrow but only when marrow samples were from individuals suffering anemic stress.

DISCUSSION

To date, most of the biochemical analyses of human erythropoiesis in vitro have dealt with the globin mRNA or protein synthetic ability of erythropoietin-unresponsive cells, such as the reticulocyte. In order to study much earlier molecular events in erythroid differentiation, e.g., the decision-making process involved in determining hemoglobin phenotype, we have cultured the early committed erythropoietin-dependent erythroid progenitor cells BFU-E and CFU-E from human marrow and BFU-E from peripheral blood. Independent biochemical techniques for the determination of globin and hemoglobin each strongly suggest that the synthesis of γ-globin and HbF is extremely low in normal human marrow cultures irrespective of erythropoietin concentration employed (Table 1). In contrast, a high level of HbF synthesis was always observed in peripheral blood BFU-E cultures from the same normal individuals. It is important to note that substantial HbF synthesis was detected in marrow cultures of two patients with SCA and one with TEC. This indicates that our cell culture system does not select
against HbF synthesis by human marrow cells. We therefore conclude that the hemoglobinized progeny of normal marrow erythroid progenitors exhibit the same pattern of hemoglobin synthesis in vitro and in vivo.

The results reported here appear to be in partial disagreement with previously published data. Papayannopoulou et al.\textsuperscript{11} have reported significant levels of $\gamma$-chain synthesis in 7–8-day plasma cultures of normal bone marrow erythroid progenitors. A number of technical differences between the two studies may be important. The survey of “normal” marrow erythropoiesis of Papayannopoulou et al.\textsuperscript{11} included many patients with lung cancer who may have been under anemic stress or whose erythroid progenitors may have been influenced by the products of their tumor. Their method of marrow collection was thoracotomy, while in this study, marrow samples were aspirated from the iliac crest. Despite these experimental variables, quantitative disagreement between the two studies is not large. For example, the average value of $\gamma/\gamma + \beta$ in the above paper\textsuperscript{11} (see their Table I) is actually 7.1% if one excludes the one individual of phenotype HbSS. Since Papayannopoulou et al.\textsuperscript{11} have recently reported that peripheral blood BFU-E-derived colonies from five normal individuals synthesized an average of 14.8% HbF, it appears that our data is actually fairly consistent with their findings, i.e., normal blood BFU-E-derived colonies synthesize far more HbF than the erythroid colonies derived from marrow erythroid progenitors. Quantitation of HbF synthesis by the progeny of human marrow erythroid progenitors grown in the methylcellulose culture system\textsuperscript{8} has led to rather different conclusions. These data are in partial agreement with ours in that normal marrow CFU-E-derived colonies synthesized less than 2% HbF, while normal peripheral blood BFU-E derived colonies made 14%–15% HbF. However, in the above study, normal marrow BFU-E-derived colonies, labeled on days 12–14, actually synthesized a greater proportion of HbF than did normal peripheral blood BFU-E. It is important to note that three of the four “normal” marrows in the above survey were from individuals under mild anemic stress and thus might more fairly be compared to our study of marrow erythropoiesis in TEC (Table I). Kidoguchi et al.\textsuperscript{8} have also found a strong positive correlation between the level of HbF synthesis and erythropoietin concentration in cultures of normal blood erythroid progenitors. We have not found any correlation between erythropoietin concentration and HbF synthesis in the erythroid colonies derived from either normal or abnormal erythroid progenitors\textsuperscript{14,32} (Table 1). Divergent results in experiments of this type can be explained by hypothesizing the existence of a “fetal factor” that may be in the commercial erythropoietin preparations used by Kidoguchi et al.\textsuperscript{8} but absent in the human urinary erythropoietin employed in our studies. Since the erythropoietin preparations used by virtually all workers in this field are less than 0.1% erythropoietin chemically,\textsuperscript{33} direct evidence that erythropoietin is either involved or not involved in the regulation of HbF synthesis cannot be easily obtained at this time.

Some of the most important work concerned with hemoglobin switching in vitro has been that of Barker and colleagues.\textsuperscript{6,7} These workers have shown that the majority of the goat marrow erythroid progenitors, whose progeny synthesize HbC in response to high concentrations of erythropoietin in culture, are probably relatively mature BFU-E and CFU-E. Although erythropoietin appears to directly control HbA to HbC switching in adult sheep and goats, recent results from the
same laboratory\(^3\) indicate that factor(s) in addition to erythropoietin may affect commitment to HbC synthesis in fetal sheep. Evidence that cellular or humoral factor(s) present in the marrow of adult sheep are important in the control of HbC synthesis\(^3\) supports this conclusion. In addition, there is now a considerable literature indicating that the normal differentiation of BFU-E to CFU-E in cultured murine marrow,\(^36,37\) human marrow,\(^38,39\) and human blood\(^40\) is absolutely dependent on factor(s) other than erythropoietin. Consideration of these new findings,\(^3\) the data we have presented above, and the fact that high erythropoietin levels in humans are often not associated with increased synthesis of HbF in vivo\(^4\) lead us to conclude that erythropoietin does not directly control the programming of human erythroid progenitor cells to varying levels of fetal hemoglobin synthesis.

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