Cultured Erythroid Cells as a Model for Hb Regulation: 
Ability of Cultured Cells to Synthesize Hb Lepore and 
HbA₂ and to Maintain Balanced Globin Synthesis

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To be useful in the study of mechanisms controlling hemoglobin synthesis, erythroid bursts cloned from peripheral blood burst-forming units (BFU-E) must be shown to perform functions characteristic of bone marrow erythroid cells. To this end, peripheral blood BFU-E were cloned from two carriers of Hb Lepore. This globin chain has the N-terminal sequence of the δ chain and C-terminal sequence of the β chain. Reticulocytes from carriers of Hb Lepore do not synthesize the δβ chain and also synthesize an excess of α chain. In contrast, nucleated erythroid cells from bone marrow of Hb Lepore carriers can both synthesize the δβ chain and maintain balanced synthesis of the α and non-α chains. Like bone marrow cells, colonies derived from BFU-E of the Hb Lepore carriers incorporated 3H-isoleucine into a peak that cochromatographed with the δchain and contained 9%-16% of the non-α chain counts.

The development of erythroid clonal cell culture techniques offers the opportunity to study the regulation of hemoglobin (Hb) synthesis in differentiating erythroid cells. However, before the in vitro culture system can be accepted as a model for in vivo development, it should be demonstrated that the cultured cells have biosynthetic and regulatory capabilities similar to those described in erythroid cells derived from bone marrow. For this purpose, we have observed the type and quantity of globin chains synthesized in vitro by erythroid bursts cultured from peripheral blood burst-forming units (BFU-E) of two carriers of Hb Lepore Washington-Boston. We wished to determine whether the cultured cells, like nucleated bone marrow cells, have the ability to synthesize the δ and δβ chains and also whether they can maintain balanced synthesis of the α and non-α globin chains.

Synthesis of both the δ chain of HbA₂ and the δβ chain of Hb Lepore occurs almost exclusively in erythroid bone marrow cells; reticulocytes have lost the ability to synthesize these chains. There is some evidence that the mRNAs that code for these chains are unstable and may be degraded before the erythroid cell reaches the reticulocyte stage. Thus, if cultured cells can synthesize the δ and δβ chains, this system may provide some insight into the mechanism underlying the instability of the δ and δβ mRNAs.

Globin chain synthesis is balanced in bone marrow erythroid cells from Hb Lepore carriers, although there is a significant excess of α chains synthesized in reticulocytes. The mechanisms that maintain balanced synthesis in bone marrow cells have not been conclusively determined, but there is some evidence for proteolysis of the excess α chains. Thus, if cultured cells also maintain balanced synthesis, this system may prove useful in further delineating the balancing mechanisms that are active in bone marrow cells.

MATERIALS AND METHODS

Informed consent was obtained from two carriers of Hb Lepore, and peripheral blood specimens were collected in ethylenediaminetetraacetic acid. Hematologic data were obtained by standard methods with a Coulter counter, model S. Hemoglobin electrophoresis, globin chain electrophoresis, and quantitations of Hb Lepore, HbA₂, and HbF were performed by standard methods.

The chromatographic separation of the Hb Lepore was performed on DEAE Sephadex resin by the method of Huisman and Dozy. Globin chains were isolated by the technique of Clegg et al., aminooethylated, and digested with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin. The tryptic peptides were separated on Aminex A-5 (Bio-Rad, Rockville Center, N.Y.), and unresolved peptides were rechromatographed on Dowex 50 × 2. Amino acid compositions of isolated peptides were determined on a Beckman Model 121 amino acid analyzer by the method of Spackman.

Globin chain synthesis in reticulocytes was performed as previously described. Peripheral blood cells were washed, and the...
red cells were incubated for 2 hr with \(^3\)H-leucine plus a mixture of amino acids, vitamins, magnesium, and iron. The cells were then washed to remove unincorporated radioactivity and lysed with deionized water, and the hemolysates were stored at \(-20^\circ\)C. After thawing, approximately 50 mg of globin was precipitated in acetone with 1% HCl at \(-20^\circ\)C. The globin chains were then separated by chromatography on carboxymethylcellulose (CMC) by using a modification of the method of Clegg et al.\(^{21}\) A nonlinear gradient was formed with three chambers containing 150 ml each of 5 mM, 10 mM, and 40 mM phosphate buffers made up in 8 M urea, 50 mM 2-mercaptoethanol. Fractions of 4 ml were collected, and 1 ml from each fraction was mixed with 10 ml of Scintisol (Isolab, Akron, Oh.) and counted for 10 min. The biosynthetic ratios were determined by integrating the counts in each peak after correcting for background counts.

Globin chain synthesis studies were also performed in cells derived from peripheral blood BFU-E cloned in methylcellulose cultures.\(^{22}\) Peripheral blood was drawn in heparin without preservative, and mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, N.J.).\(^{22}\) These cells were then plated in methylcellulose by the method of Iscove et al.\(^{27}\) as modified by Ogawa et al.\(^{28}\) except that bovine serum albumin was purchased from Sigma (St. Louis, Mo.), and 2 \times 10^5 cells were plated in 35 \times 10 mm Falcon Petri dishes. Three different lots of fetal bovine serum (FBS) were used when patient B.C.’s cells were plated. After 12 days in culture, 100 \mu Ci of \(^3\)H-leucine were added to each plate and the plates were incubated for 48 hr. The erythroid bursts were then harvested and washed to remove unincorporated \(^3\)H-leucine. The cells were lysed with 0.2 ml of deionized water. Approximately 20 mg of unlabeled carrier hemolysate was added, and hemolysates were stored at \(-20^\circ\)C. After thawing, the globin chains were precipitated in acetone-HCl and purified by gel filtration chromatography on Sephadex G-100; the cells were lysed, 20 mg of unlabeled carrier hemolysate was added, and the hemolysates were stored at \(-20^\circ\)C. After thawing, the globin chains were precipitated in acetone-HCl and purified by gel filtration chromatography on Sephadex G-100 in 20% HCOOH. The globin fractions were pooled and lyophilized, and the globin chains were then separated as described above.

The y chain is the only globin chain that contains isoleucine. Therefore, to determine whether the cultured cells produce nonglobin proteins that might cochromatograph with the \(\delta\beta\) or \(\delta\) chain, we added 100 \mu Ci of \(^3\)H-isoleucine to one plate. After 48 hr, the erythroid bursts were harvested as described above. To maximize the opportunity to observe contaminants, the globin was not purified on Sephadex G-100; the cells were lysed, 20 mg of unlabeled carrier hemolysate was added, and the globin chains were precipitated and separated as described above.

To analyze the purified hemoglobin synthesized by the cultured cells, HbS \(\alpha_2\) Lepore, and \(\alpha + \) F were isolated by chromatography on a 0.6-cm \times 16-cm column of DE-52 equilibrated with 0.05 M Tris and 0.1 g/dl KCN, adjusted to pH 8.5.\(^{15}\) HbA\(_2\) was removed with the same buffer adjusted to pH 8.35. Hb Lepore was eluted with pH 8.2 buffer, and the remaining Hb was then eluted with pH 7.0 buffer.\(^{16}\) Each Hb solution was then concentrated and dialyzed against deionized water for 48 hr to remove the Tris and KCN. Purified HbA\(_2\) carrier was added to the HbA\(_2\), and whole hemolysate was added to the Hb Lepore and to the HbA + F. For each Hb type, the globin chains were then analyzed as previously described.

**RESULTS**

**Subjects**

B.C. is a 49-yr-old black woman with a history of mild microcytic anemia. She had an Hb level of 12.6 g/dl, red cell count of 6.7 \times 10^{12} cells/liter, mean cell volume (MCV) of 73 fl, mean cell hemoglobin (MCH) of 22.9 pg, and mean cell hemoglobin concentration (MCHC) of 31.5 g/dl. Cellulose acetate electrophoresis at pH 8.6 revealed an Hb variant that migrated at the HbS position and comprised 12% of the total hemoglobin. Citrate agar electrophoresis at pH 6.1 showed only one band at the HbA position. The variant comigrated with a known Lepore on globin chain electrophoresis. Her HbA\(_2\) level was 2.3%, and her HbF level was 1.5%.

M.O. is a 28-yr-old woman of Mexican descent. Hematologic analysis showed an Hb level of 12 g/dl, red cell count of 5.7 \times 10^{12} cells/liter, MCV of 66 fl, MCH of 20.9 pg, and MCHC of 31.6 g/dl. Electrophoretic results were similar to those of B.C., with a variant migrating in the HbS position which comprised 13% of the total hemoglobin. Her HbA\(_2\) level was 2.2% and her HbF level was 3.2%.

**Structural Analysis of the Variant Chain**

The total amino acid composition of the abnormal chain from each subject suggested a Washington-Boston fusion chain. To confirm this finding, the variant chain from M.O. was isolated and digested with trypsin. The tryptic peptides were separated by ion exchange chromatography, and their compositions were determined by amino acid analysis. All of the peptides characteristic of \(\delta\beta\) Washington-Boston were clearly evident except for \(\delta\) or \(\beta\) tryptic peptides TpX and XI. The \(\delta\) chain differences were present in positions 9(Thr), 12(Asn), 22(Ala), and 50(Ser) as compared to the normal chain. The amino acid at position 116 was histidine, indicative of \(\beta\) chain. The rechromatography of the mixture of \(\delta\) or \(\beta\) TpX and XI revealed the presence of 6 TpX and \(\delta\) or \(\beta\) TpXI. Thus, the abnormal chain has a \(\delta\) chain sequence to position 87, \(\delta\) and \(\beta\) to position 115, and \(\beta\) from position 116 to the C-terminal end, as described by Baglioni\(^{31}\) and found in many unpublished cases from this laboratory.

**Biosynthesis of the \(\delta\beta\) Chain**

Globin labeled with \(^3\)H-leucine. Figure 1 depicts representative chromatograms obtained from analysis of globin chains synthesized by BFU-E derived from a normal control, by erythroid colonies derived from BFU-E from B.C., and by reticulocytes from B.C. Virtually no synthesis of the \(\delta\beta\) chain was observed in the reticulocyte system. However, in the erythroid colony system, we observed a peak of radioactivity that cochromatographed with the \(\delta\beta\) chain. This peak contained 9%–16% of the total non-\(\alpha\) chain counts, consistent with the production of \(\delta\beta\) chain observed in bone marrow studies.\(^{3,5,6}\) Erythroid colonies from normal BFU-E also synthesized a radioactive peak in the \(\delta\beta\) position, but this comprised less than 4% of the
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Fig. 1. Incorporation of ³H-leucine into the globin chains synthesized by erythroid bursts cultured from peripheral blood BFU-E from a normal subject (top panel), by erythroid bursts cultured from peripheral blood BFU-E from B.C. (middle panel), and by peripheral blood reticulocytes from B.C. (bottom panel). The reticulocytes from B.C. synthesized almost no δ chain, and the α/δβ synthesis ratio was elevated. In contrast, cells cloned from BFU-E from B.C.’s peripheral blood synthesized the δβ chain, and their α/α-non-α synthesis ratio was similar to ratios obtained in bursts cloned from normal BFU-E.

Fig. 2. Incorporation of ³H-isoleucine into the globin chains synthesized by erythroid bursts cloned from peripheral blood BFU-E from B.C. The γ chain is the only globin chain that contains isoleucine, so any counts found in the other globin chains can be considered contaminants. Although this globin was not purified by gel filtration chromatography on Sephadex G-100, only small amounts of radioactivity were associated with the non-γ chains.

Non-α chain counts and possibly represents δ chain synthesis.

Globin labeled with ³H-isoleucine. Since the radioactive peak in the δβ position could possibly be an artifact that was not truly δβ chain, we labeled one plate from B.C. with ³H-isoleucine. Since isoleucine is found in the γ chain, but not in α, β, or δβ chains, incorporation of ³H-isoleucine into any of the non-α globin peaks would represent contamination by some other protein that contains isoleucine. The level of contamination was maximized by not purifying the globin on Sephadex G-100 before CMC chromatography. The result of this experiment is shown in Fig. 2. The small radioactive peak in the δβ position contains only about 10% of the counts incorporated into the γ chain. In contrast, when B.C.’s plates were labeled with ³H-leucine as described above, more radioactivity was incorporated into the δβ chain peak than into the γ chain peak. Thus, although we cannot rule out the possibility of a contaminant that does not contain isoleucine, it seems unlikely that a contaminant protein could account for the ³H-leucine incorporated into the δβ chain. In addition, relatively few counts were incorporated into the non-γ chain positions when the cultures were labeled with ³H-isoleucine, suggesting that this system synthesizes little isoleucine-containing protein that cochromatographs with the non-γ globin chains.

Purified Hemoglobins

Since the δβ chain elutes with the δ chain on CMC chromatography, we wished to be certain that the radioactive peak in the δβ position was not due solely to δ chain synthesis. Therefore, we separated the different Hb types on DE-52 before CMC chromatography.

Figure 3 shows chain separations of the hemoglobins previously isolated on DE-52. The purified HbA₂ contained two large radioactive peaks, one in the δ chain position and one in the α chain position. The purified Hb Lepore also contained two major radioactive peaks, one in the δ position, which is δβ chain, and one in the α position. The small radioactive peak in the β position suggests that a small amount of HbA (approximately 2%) was eluted with the Hb Lepore. The purified HbA + HbF contained major radioactive peaks in the α and β positions plus a small peak in the γ position.

Globin Synthesis Ratios

The synthesis ratios obtained from experiments with both reticulocyte and erythroid colony synthesis are summarized in Table 1. In both B.C. and M.O.,
globin chain synthesis was unbalanced in reticulocytes, and no significant synthesis of the δβ chain was observed. In the culture system, colonies derived from BFU-E from both B.C. and M.O. synthesized the δβ chain, and globin chain synthesis was balanced. The five sets of data listed for B.C.'s erythroid cultures represent experiments using 3 lots of FBS. The first two sets were duplicate cultures using the same lot of FBS that was used in the other experiments described. The next two sets were duplicate cultures using a second lot of FBS, and the fifth set used a third lot of FBS. The first lot of FBS was more effective than the other two lots in promoting erythroid growth. However, similar synthesis ratios (α/β + δβ + γ and δβ/β + δβ + γ) were observed in all five sets of data.

The balanced synthesis described above was confirmed by chain separations of the component hemoglobins synthesized by erythroid bursts from B.C. Both the purified Hb Lepore and the purified HbA + HbF showed balanced α/non-α synthesis ratios. The slight excess of α chains in the purified HbA2 may represent contamination of the NβA2 by a small pool of radioactive α chain dimers. Calculations of synthesis ratios from these studies showed a total α/β + δβ + γ ratio of 1.09, with γ, δ, δβ, and β chains, respectively, comprising 5%, 2%, 9%, and 84% of the non-α chain synthesized. These percentages are in close agreement with results obtained from analysis of the total globin chain synthesized. The balanced synthesis confirms the ability of cultured cells to prevent the accumulation of excess α chains. However, the cells may be synthesizing more α than non-α chains, but over the long labeling period, the synthesis and degradation of excess α chains may have reached an equilibrium.

### Table 1. Globin Chain Synthesis in Reticulocytes and in Erythroid Bursts Cloned From Peripheral Blood BFU-Es From Two Carriers of Hb-Lepore

<table>
<thead>
<tr>
<th>Subject</th>
<th>Reticulocytes</th>
<th>Erythroid Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Counts × 10^3</td>
<td>Synthesis Ratio</td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td>M.O.</td>
<td>44.53</td>
<td>22.32</td>
</tr>
<tr>
<td>B.C.</td>
<td>46.26</td>
<td>24.31</td>
</tr>
<tr>
<td>B.C.</td>
<td>146.45</td>
<td>7.52</td>
</tr>
<tr>
<td>B.C.</td>
<td>15.06</td>
<td>1.84</td>
</tr>
<tr>
<td>B.C.</td>
<td>12.21</td>
<td>1.65</td>
</tr>
<tr>
<td>B.C.</td>
<td>4.05</td>
<td>1.26</td>
</tr>
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</table>

The five sets of erythroid cultures listed for B.C. represent experiments using 3 different lots of fetal bovine serum (FBS). The first two sets of data were obtained with the same lot of FBS used in the other experiments described. The third and fourth sets were obtained with a second lot of FBS, and the fifth set was obtained with a third lot of FBS. The first lot of FBS was most effective in supporting erythroid growth. However, the α/ non-α and δβ/non-α synthesis ratios were similar in all five cases.

*δβ chain counts also include δ chain counts.
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DISCUSSION

Since the offspring of cultured peripheral blood BFU-E can synthesize the $\delta\beta$ chain of Hb Lepore and the $\delta$ chain of HbA2, this system may provide some insight into the mechanism for the early termination of both $\delta\beta$ and $\delta$ chain synthesis. The finding of increasing $\beta/\delta\beta$ synthesis ratios as erythroid cells mature suggests that $\delta$ chain mRNA may be less stable than $\beta$ chain mRNA.\(^1\)\(^2\)\(^5\)\(^9\) Similarly, $\beta/\delta\beta$ synthesis ratios increase as erythroid cells from carriers of Hb Lepore mature, suggesting instability in the $\delta\beta$ mRNA.\(^3\)\(^5\) In addition, $\delta/\alpha$ synthesis ratios decrease as erythroid cells from homozygotes for Hb Lepore mature.\(^7\)\(^8\)

Several investigators have directly demonstrated "$\beta$-like"/$\alpha$ mRNA ratios of only 0.01–0.03 in peripheral blood cells from homozygotes for Hb-Lepore.\(^7\)\(^10\) Since these homozygotes had some nucleated cells in their peripheral blood, even these low levels of $\delta\beta$ mRNA may be derived from nucleated cells rather than from reticulocytes. In contrast to the findings in peripheral blood, Giglioni et al. have demonstrated that bone marrow cells from persons homozygous for $\beta$ thalassemia have "$\beta$-like"/$\alpha$ mRNA ratios of 0.06–0.08.\(^9\) The mechanism for the loss of the $\delta$ and $\delta\beta$ mRNAs as erythroid cells mature is unknown. Since the cultured cells can produce and translate both the $\delta$ and $\delta\beta$ mRNAs, this system may prove useful in elucidating the reason for the turnover of the $\delta$ and $\delta\beta$ mRNAs.

In addition to the instability of their RNAs, the synthesis of both the $\delta$ and $\delta\beta$ chains appears to be limited by inefficient transcription and/or processing of the mRNA precursors for these chains. Even early erythroid cells synthesize low levels of $\delta$ and $\delta\beta$ chains relative to $\beta$ chains.\(^1\)\(^3\)\(^5\) Bone marrow cells from homozygotes for $\beta$' thalassemia have "$\beta$-like"/$\alpha$ mRNA ratios of only 0.02–0.03,\(^9\) suggesting that defects in transcription or processing of mRNA precursors may be a major constraint on the production of the $\delta$ chain. Recent experiments using $^{32}$P-labeled DNA probes specific for the $\delta$ or $\beta$ introns show a lower concentration of $\delta$ mRNA precursor than $\beta$ mRNA precursor in bone marrow cells from persons homozygous for $\beta$' thalassemia.\(^34\) This suggests a defect in transcription or early processing of the $\delta$ chain mRNA precursor. Studies of in vitro transcription in a HeLa-derived cell-free system indicate that the $\delta$ chain mRNA precursor is less efficiently transcribed than the $\beta$ chain mRNA precursor.\(^35\) Cultured erythroid cells may prove useful in sorting out the relative contributions of inefficient transcription, defective processing, and instability of the mRNA to the relatively low production of the $\delta$ and $\delta\beta$ chains.

Cultured cells may also be a useful system for the study of mechanisms that balance $\alpha$ and non-$\alpha$ chain synthesis. Several investigators have pointed out that globin chain synthesis is more balanced in bone marrow cells than in peripheral blood reticulocytes of persons with $\beta$ thalassemia trait\(^32\)\(^33\) or Hb Lepore trait.\(^3\)\(^6\) Although the balancing mechanism has not been conclusively determined, proteolysis of the excess $\alpha$ chains has been suggested.\(^1\)\(^11\) We have obtained lower $\alpha$/$\alpha$-$\beta$ ratios in cultured cells than in reticulocytes from both carriers of Hb Lepore, which suggests that the bone marrow balancing mechanisms are active in the cultured cells. Thus, the culture system may provide some insight into the operation of the balancing mechanisms.

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