Globin Synthesis in Mouse Erythroleukemia Cells In Vitro: A Switch in \( \beta \) Chains Due to Inducing Agent

By Blanche P. Alter and Sabra C. Goff

Friend erythroleukemia cells, originally derived from DBA/2 mice, differentiate when cultured with inducing agents. Studies of the different effects of inducing agents on clone 745 have revealed that both dimethyl sulfoxide (DMSO) and hemin produce benzidine-positive cells. Butyric acid produced mature but benzidine-negative cells in this clone. All agents induced globin synthesis above the 0.1% of protein synthesis found in uninduced cells. DMSO induction stimulated globin synthesis 9%, hemin 2%, and butyric acid 3%. Total \( \beta/\alpha \) ratios were approximately unity with all agents. Although the inducing agents all stimulated total globin synthesis in Friend cells, the relative rates of synthesis of the two \( \beta \) chains were affected differently by the various agents. Hemin markedly increased the proportion of \( \beta \) minor. For example, DBA/2 mouse reticulocytes synthesized 20% \( \beta \) minor and 80% \( \beta \) major. DMSO induction of clone 745 caused 20%–33% synthesis of \( \beta \) minor, and butyric acid 30%–37% \( \beta \) minor. In contrast, hemin increased the proportion of \( \beta \) minor to 64%–69%. Thus the Friend erythroleukemia cell system provides an in vitro approach to the study of the regulation of globin-chain switching.

In man, replacement of fetal \((\alpha_2\gamma_2)\) by adult \((\alpha_2\beta_2)\) hemoglobin occurs during normal development. This switch is incomplete in many hematologic disorders. Patients with hemoglobinopathies such as sickle cell disease or \( \beta \)-thalassemia have fewer symptoms if some fetal hemoglobin synthesis is retained. The specific regulatory factors that control the fetal-adult switch are not known.

We describe here an in vitro model for this switch. We have used the mouse erythroleukemia cell system that grows in vitro\(^1,\,2,\,4\) and matures when dimethyl sulfoxide (DMSO) is added.\(^3\) Boyer et al.\(^5\) demonstrated that these cells synthesize adult mouse globin chains. DBA/2 mice, in which the cells originated, have \( \beta \)-chain heterogeneity, with 80% major and 20% minor hemoglobin.\(^6\) The minor \( \beta \) chain differs from the major by nine amino acid residues.\(^7,\,8\)

Ostertag et al.\(^9\) reported that the \( \beta/\alpha \) ratio was 1 in FSD-1/c14, but 3 in FSD-1. Kabat et al.\(^10\) found the ratio to be 1 in FSD-1 and in clone 745. They detected 40% \( \beta \) minor in clone 745, but only 10% in FSD-1. Both groups studied the effect of a single agent, DMSO, on different clones.

Other inducing agents also lead to the maturation of Friend cells. Leder and Leder\(^11\) described the use of butyric acid. Harrison et al.\(^12\) and Ross and

From the Division of Hematology and Oncology, Children's Hospital Medical Center, Sidney Farber Cancer Institute, and the Department of Pediatrics, Harvard Medical School, Boston, Mass.

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Address for reprint requests: Blanche P. Alter, M.D., Division of Hematology and Oncology, Children's Hospital Medical Center, 300 Longwood Avenue, Boston, Mass. 02115.

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Sautner\textsuperscript{11} employed hemin. Globin-chain analyses have not been reported previously with these agents. In the studies reported here we investigated a single clone, 745, utilizing three different inducing agents. Our data showed balanced globin synthesis with all inducing agents, but the proportion of $\beta$ minor synthesis depended on the particular inducing agent. Induction of Friend cell clone 745 with DMSO or butyric acid stimulated $\beta$ major more than $\beta$ minor, while induction with hemin resulted in a predominance of $\beta$ minor production. Thus the type of globin chain produced depended on the chemical environment of the cell and was subject to manipulation in vitro. In addition, marked differences in the morphology of the induced cells resulted from varying effects of the inducing agents on the concomitant expressions of heme and globin synthesis.

**MATERIALS AND METHODS**

Friend cell clone 745 was the kind gift of Dr. Albert Deisseroth, who had originally obtained it from the Institute for Medical Research, Camden, N.J. It differed in its induction by butyric acid from clone 745 cells which we obtained directly from the Institute for Medical Research.

Cells were maintained in suspension in Dulbecco's modification of Eagle's medium, with 10% heat-inactivated fetal calf serum (Flow Laboratories), 2 mM glutamine, 100 $\mu$g/ml streptomycin and penicillin, and 0.25 $\mu$g/ml Fungizone. The cells were grown in a humidified water-jacketed incubator, with 10% CO$_2$. The cells were passed twice weekly and were plated at 5 x 10$^5$ cells/ml. A single lot of serum was used.

For induction, cells were transferred at 10$^3$ cells/ml into medium containing the inducing agent. DMSO was obtained from Fisher Chemical Co. and was not filtered prior to use. Final concentration was 2% (280 mM). Hemin (Eastman Organic Chemicals) was dissolved according to the method of Adamson et al.\textsuperscript{14} and filtered through a 0.22-$\mu$m filter (Millipore). The concentration was then determined by the absorbance at 557 nm.\textsuperscript{15} The final concentration in most experiments was 75 $\mu$M. Butyric acid (Aldrich Chemical Co.) was added to medium in a chemical fume hood. The medium was then filtered through a 0.22-$\mu$m filter in some experiments, and it was unfiltered in others; filtration did not influence the results. The final concentration of butyric acid was 1 mM.

Cell counts were done with a hemocytometer. Cytoslides were made with the Shandon cytocentrifuge and were stained with benzidine Wright-Giemsa.

Cells were labeled with $^3$H-leucine for 4-hr pulses on the days indicated, usually 1 day before maximal morphologic change. The cells from 10 ml of culture medium were centrifuged and then resuspended in 2 ml of medium which lacked leucine and contained fetal calf serum which had been dialyzed against Hanks' balanced salt solution. Then 100 $\mu$Ci of $^3$H-leucine, 80 Ci/mole (New England Nuclear Corp.) was added to each 2-ml suspension. After 4 hr at 37°C, the cells were harvested by centrifugation, washed three times in Hanks' balanced salt solution containing 1% bovine serum albumin, and lysed in 50 $\mu$l of 1 mM phosphate containing 0.5% Nonidet-P40. DBA/2J adult mouse hemoglobin was added as carrier. After 15 min of lysis at 37°C, 25 $\mu$l of CCl$_4$/toluene (2/1) was added, the samples vortexed, and stroma removed by centrifugation. This method led to complete lysis of nucleated cells, and did not cause loss of significant amounts of hemoglobin.

For the determination of total protein synthesis, an aliquot was added to 1 ml of 0.1 N NaOH. The hydrolysates were incubated at 37°C for 20 min to break the $tRNA$-leucine bond, ensuring that the material subsequently precipitated with 5% trichloroacetic acid (TCA) would be only protein. The TCA precipitates were filtered on 0.45-$\mu$m filters (Matheson-Higgins Co.), washed five times with 5% TCA, and dried. The filters were then placed in 10 ml of Aquasol (New England Nuclear Corp.), or Instagel (Packard Instrument Co.) and left overnight. The filters dissolved, and the samples were counted at 30% efficiency in a Packard liquid scintillation counter.

The pattern of protein synthesis was analyzed by electrophoresis of the lysates on sodium dodecyl sulfate (SDS) 10% polyacrylamide gels in urea as previously described by Alter and Ingram.\textsuperscript{16} The proportion of net globin synthesis was determined by subtracting the gel back-
ground in the globin region, adding the remaining radioactivity in the globin region, and divid-
ing this "net globin" radioactivity by the total radioactivity on the gel.

For the recovery of globin from gels, lysates were electrophoresed as described, unstained gels were quickly scanned in water at 280 nm on a Gilford model 2400 spectrophotometer, and the globin region was sliced into 1-mm slices, eluted into deionized water, and then lyophi-
lized.16 Recoveries were approximately 90%. This material of molecular weight in the range of globin was dissolved in starting buffer for chromatography (3.75 mM phosphate in 8 M urea, pH 6.9) on carboxymethylcellulose (CMC) columns (CM52, Whatman); 20 mg of unlabeled DBA/2J globin was added as carrier. Chromatography was done with a modification of the methods de-
scribed by Clegg et al.17 A nonlinear gradient was established with an LKB Ultragrad gradient-
forming device. The final buffer was 35 mM phosphate. Sixty-five fractions of 7 ml were collected.

Absorbances were measured at 280 nm, using a rapid sampling device on a Gilford model 2400 spectrophotometer. Radioactivity was determined by pipetting 1 or 3 ml of each fraction into 10 ml of Instagel and counting with 30% efficiency in a Packard model 3375 liquid scintillation counter. Counting was done to 2% counting error. Chain ratios were determined by drawing a baseline from the low points surrounding each globin peak and then summing the net counts in each peak. The proportion of globin-chain radioactivity on each column was calculated by adding the net counts in each of the globin peaks and dividing by the total counts recovered from the column. Recovery of pure globin was over 90% on these columns.

The overall proportion of total protein synthesis which was globin chains was determined by multiplying the percent net globin on the SDS polyacrylamide gels by the percent of this ma-
terial which cochromatographed with globin chains on the CMC columns. In this way, proteins of the globin molecular weight range (16,000 daltons) were further quantified by ion-exchange chromatography.

In some experiments, cells were incubated with 35S-methionine. After electrophoresis and chromatography, the globin peak tubes were pooled, dialyzed against 0.5% formic acid, lyophi-
lized, fingerprinted on 0.1-mm cellulose thin-layer plates (Brinkmann), and autoradiographed as previously described.16

RESULTS

Culture of our line of Friend cell clone 745 with the inducing agents produced the morphologic changes shown in Fig. 1. Uninduced cells, shown in Fig. 1A, resembled proerythroblasts and remained benzidine-negative. After 5 days in 2% DMSO, the cells were smaller and there were many polychroma-
tophilic and orthochromatic erythroblasts (Fig. 1B). Nuclear chromatin was diffuse. Culture for 5 days in 75 μM hemin led to small cells with dark benzidine-
positive cytoplasm (Fig. 1C). Treatment with 1 mM butyric acid led to small cells, pyknotic nuclei, and pale cytoplasm (Fig. 1D); they were benzidine-
negative.

By contrast, several other clones of Friend cells were all found to become benzidine-positive with butyric acid. These included T3C12, 707, and a different strain of clone 745 which was recently purchased from the Institute for Medical Research. Thus the morphology described above was unique to our particular line of clone 745.

Despite the variable morphologic changes induced in Friend cells, all agents stimulated globin synthesis. Figure 2 shows the results of electrophoresis of radioactive lysates on SDS–polyacrylamide gels. In the lysate from uninduced cells, less than 1% of newly synthesized protein electrophoresed in the globin region. DMSO led to 18% apparent globin, while lysates from hemin and butyric acid–treated cells had 6% globin in the experiment shown here. Table 1 outlines these results at daily intervals during induction and demonstrates an
Fig. 1. Photomicrographs of clone 745 Friend cells. Cytocentrifuge slides were stained with benzidine Wright-Giemsa. × 1000. (A) Uninduced cells. (B) Cells following induction with 2% DMSO for 5 days. (C) Cells following induction with 75 μM hemin for 5 days. (D) Cells following induction with 1 mM butyric acid for 3 days.

Fig. 2. SDS-polyacrylamide gel electrophoretic patterns of proteins synthesized in clone 745 Friend cells. Cells were incubated with 3H-leucine for 4 hr on the days indicated. i, globin region. (A) Uninduced cells, on day 2. (B) With 2% DMSO, on day 4. (C) With 75 μM hemin, on day 3. (D) With 1 mM butyric acid, on day 2.
Table 1. Induction of Globin-Chain Synthesis in Clone 745

<table>
<thead>
<tr>
<th>Inducing Agent</th>
<th>Net &quot;Globin&quot;</th>
<th>$\beta + \alpha$</th>
<th>Overall $\beta$ Minor</th>
<th>Overall $\beta$ Major</th>
<th>Overall $\beta$ Major + Minor</th>
<th>Overall $\beta$ Minor + $\beta$ Major</th>
<th>Overall $\beta$ Minor</th>
<th>Morphologic Maturation $\beta$</th>
<th>Net &quot;Globin&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.9</td>
<td>4.9</td>
<td>0.04</td>
<td>47</td>
<td>0.01</td>
<td>0.01</td>
<td>1.06</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>DMSO</td>
<td>2.1</td>
<td>19.6</td>
<td>0.41</td>
<td>20</td>
<td>0.14</td>
<td>0.03</td>
<td>0.78</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Hemin</td>
<td>3.9</td>
<td>31.9</td>
<td>1.23</td>
<td>64</td>
<td>0.22</td>
<td>0.39</td>
<td>1.00</td>
<td>4</td>
<td>0.00</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>7.6</td>
<td>33.6</td>
<td>2.35</td>
<td>30</td>
<td>0.80</td>
<td>0.34</td>
<td>0.81</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*On SDS-polyacrylamide gels.
†On CMC columns.
‡Product of preceding two columns.
§Percent of net radioactivity ($\beta$ minor/($\beta$ major + $\beta$ minor)) on CMC column.
¶Proportion of each globin chain/total globin × overall globin (%).

Induced globin synthesis measured by benzidine-positive cells with DMSO or hemin, benzidine-negative small cells with butyric acid.

apparent stimulation in globin production by each of the agents at all times studied.

Material of the globin molecular weight range was recovered from the SDS-polyacrylamide gels and chromatographed on CMC columns (Fig. 3). The uninduced cells had a small amount of material which cochromatographed with globin and which comprised less than 0.1% of total protein synthesis (see Materials and Methods for this calculation).

DMSO induction led to a pattern of globin chains which resembled that of the carrier DBA/2J globin. Overall, a maximum of 9% of newly synthesized protein was found to be globin. With hemin induction 2% was globin, and with
butyric acid 2%-3% was globin. These values are shown in Table 1 for several time points.

In a different experiment, 35S-methionine was used to label induced cells. The putative globin chains were fingerprinted. Material from the β major region had two methionine peptides, while material from the β minor and α regions had one methionine peptide each. In all cases, the methionine peptides from the Friend cell globin peaks were identical to the methionine peptides of bona fide DBA/2J β major, β minor, and α chains. According to these fingerprint data, the relevant globin regions were approximately 90% pure.

The most striking finding on examination of Fig. 3 is that the pattern of the globin chains varied with the inducing agents. The carrier DBA/2J globin contained 20% β minor and 80% β major. DMSO treatment resulted in 25% β minor, similar to the carrier. With butyric acid, β minor was 37%. With hemin, however, β minor exceeded β major and comprised 69% of the total β chain in this experiment. Although the hemin-treated cells resembled the controls in that β minor was the predominant β chain in both, the hemin-treated cells also exhibited an absolute increment in globin production. As shown in Table 1, the relative proportion of β minor production was similar at all time points for each agent and depended on the inducing agent. In addition, the total β/α synthetic ratios were approximately 1 with all agents at all times.

Figure 4 shows that globin production was readily detectable by our methods prior to changes in the morphologic appearance of the cells. Thus, globin synthesis preceded the development of benzidine-positive cells in the DMSO or hemin-treated cultures, or benzidine-negative but pyknotic cells in the butyric acid-treated culture.

**DISCUSSION**

Our studies of the Friend mouse erythroleukemia model have revealed several unique features. We employed three different inducing agents; DMSO, hemin,
and butyric acid. In this group, only hemin has a known role in erythropoiesis in vivo. Growth in each of the inducers led to cells which varied in their morphology and in their biochemistry.

Uninduced cells of clone 745 retained the appearance of proerythroblasts. Ross et al.\textsuperscript{18,19} and Orkin et al.\textsuperscript{20} were unable to detect globin mRNA in uninduced clone T3C12 cells. We found newly synthesized globin in uninduced clone 745 cells at all times examined, although it was usually less than 0.1\% of total protein synthesis. Our method may be more sensitive than molecular hybridization analyses performed in the presence of large inputs of RNA. The differences, however, are probably due to the use of different cell lines or different culture techniques. Conkie et al.\textsuperscript{21} and Harrison et al.\textsuperscript{22} did detect globin mRNA in uninduced cells of still another clone, clone 707.

DMSO induction of clone 745 led to benzidine-positive polychromatophilic and orthochromatic megaloblasts, which began to appear after 3 days in culture, as was also observed by Friend et al.\textsuperscript{3} During DMSO induction, the proportion of protein synthesis which was globin rose rapidly and preceded the appearance of benzidine-positive cells. The biosynthetic assay for globin synthesis is undoubtedly more sensitive than is the benzidine stain for hemoglobin accumulation. Ross et al.\textsuperscript{18} detected globin mRNA at 1–2 days following induction with DMSO. Globin synthesis may thus precede heme synthesis in some situations, as suggested by Sassa.\textsuperscript{23} Nathan et al.\textsuperscript{24} did observe that globin synthesis preceded heme production in the early human erythroblast. Glass et al.\textsuperscript{25} however, detected heme synthesis in early mouse pronormoblasts, while hemoglobin was detected only later in development. Bruns and London\textsuperscript{26} observed the cessation of globin synthesis in heme deficiency. The interaction and coordination of heme and globin synthesis may vary from one system to another.

In our experiments with clone 745, DMSO induction led to a $\beta/\alpha$ ratio that was approximately 1 at all times. This finding is contrary to the report of Orkin et al.\textsuperscript{20} that the $\alpha/\beta$ globin mRNA ratio was 3 at 30 hr and decreased to 1 at 4 days; however, their studies were done with different and unrelated clones. In addition, Orkin et al. may have been detecting embryonic $\alpha$-like mRNA, while we examined adult $\alpha$-globin synthesis. Furthermore, globin synthesis may not directly reflect globin mRNA content.

DMSO induction led to 20\%–33\% $\beta$ minor production. This level is similar to the 20\% $\beta$ minor found in DBA/2 mice. Kabat et al.\textsuperscript{10} detected 40\% $\beta$ minor in DMSO-treated clone 745; our results are probably similar, since the methods of analysis differed somewhat. Kabat et al.,\textsuperscript{10} as well as Ostertag et al.,\textsuperscript{9} prepared globin from complete lysates of Friend cells, chromatographed the material on CMC columns, and subtracted the high background found in the lysates of uninduced cells from the data in the lysates of induced cells. In our method, the nonglobin proteins synthesized by the Friend cells were removed by electrophoresis on SDS–polyacrylamide gels followed by selection of only the globin molecular weight material. Further analysis on CMC columns revealed that some of this material was also nonglobin and allowed a more precise analysis of the actual globin.

Butyric acid induction of this clone of 745 led to morphologic results which
differ from those reported by Leder and Leder. In our experiments, the cells remained benzidine-negative. Despite this apparent failure to accumulate hemoglobin, the induced cells did synthesize globin chains, and both the $\beta/\alpha$ ratio of 1 and the percentage $\beta$ minor of $30\%-37\%$ resembled the globin synthesis in DMSO-induced cells. These findings further suggest that globin synthesis and heme synthesis may in fact be dissociated, as discussed above.

Clone 745 cells grown in hemin became benzidine-positive, but were small, with pyknotic nuclei, and thus differed from those induced with DMSO. Globin synthesis reached the maximum by the second day, although the cells continued to mature. The $\beta/\alpha$ ratio was approximately 1. The $\beta$-chain synthesis, however, was shifted to $\beta$ minor, which comprised almost 70% of the total $\beta$ synthesis. Since total globin synthesis was not stimulated as much with hemin as it was with DMSO (maximum of 9%), the absolute amount of $\beta$ minor produced was similar with each inducing agent, and comprised approximately 1% of protein synthesis. This amount far exceeded the amount of $\beta$ minor produced in uninduced cells (maximum of 0.06% of protein synthesis, Table 1).

There are two possible models for the different results of induction with DMSO and hemin. In the single-cell model, all cells contain the linked $\beta$ major and $\beta$ minor genes, whose relative production depends on intracellular regulatory events. In the adult DBA/2 mouse, the production rate for $\beta$ major would be 80%, and $\beta$ minor 20%. In clone 745 Friend cells induction with DMSO or butyric acid would lead to similar relative outputs. Hemin-treated or uninduced cells would be switched over toward $\beta$ minor production. This state resembles that found in the newborn human, in which linked $\gamma$ and $\beta$ genes are regulated by some developmental event. The human fetus makes mostly $\gamma$ chain, but switches over to $\beta$-chain production at birth. Various dyserythropoietic stresses cause the cells to revert to predominantly $\gamma$-chain synthesis. Weatherall et al. have recently reviewed this switch, and suggested that this regulation may not be intracellular, but may represent the selective survival of cells which are already preprogrammed for $\gamma$-chain synthesis.

According to this model, clone 745 consists of two populations of cells, one primarily or entirely responsible for $\beta$ major synthesis, and the other for $\beta$ minor. DMSO or butyric acid induces globin synthesis by the cells responsible for $\beta$ major more than those for $\beta$ minor. Hemin, on the other hand, selectively stimulates synthesis of globin by the cells programmed predominantly for $\beta$ minor.

In the above models we have compared the stimulation of $\beta$ minor by hemin with the reversion to $\gamma$-chain synthesis in the human example. The murine $\beta$ minor gene might instead be more analogous to the human $\delta$ gene, since both are present as minor non-$\alpha$ genes syntenic with $\beta$ genes in the adult animals of each species. The major human example of an increase in $\delta$-chain production occurs in patients with $\beta$-thalassemia, but our data do not suggest a "thalassemic" defect during the induction of our clone of Friend cells.

The differentiation of Friend leukemia cells in response to inducing agents provides an in vitro model for erythropoiesis. The morphology of the differentiated Friend cell, and the biochemistry of the cell following chemical induction, depend on the clone of cells as well as on the choice of inducing agent.
Thus any conclusions about erythroid differentiation must be specified in terms of the system which is being examined. Nevertheless, such studies may allow dissection of components of erythroid development and thus provide valuable information concerning the necessity for coordination of heme and globin synthesis, the relative proportion of \( \beta \)-chain and \( \alpha \)-chain production, and the regulation of non-\( \alpha \) chains, such as \( \beta \) major and \( \beta \) minor. Manipulation of specific segments of erythroid differentiation in vitro may eventually lead to therapeutic application in vivo.

We note that a recent publication by Nudel et al.\(^{28}\) also demonstrated differential induction of \( \beta \)-globin genes in Friend cells that depended on the type of inducing agent.

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