Hemoglobin Biosynthesis in Murine Virus-induced Leukemic Cells In Vitro: Structure and Amounts of Globin Chains Produced

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Tryptic peptides characteristic of mouse hemoglobin α- and diffuse-type β-chains were isolated from dimethylsulfoxide-treated mouse leukemia virus-infected cell cultures. Quantitative estimates of in vitro hemoglobin synthesis by such cultures were obtained by two different methods: by analysis of radioactive amino acid incorporation, and by single-cell radial immunodiffusion assay. The amounts produced, approximately 4 pg/cell/17 hr, reflect a synthetic capacity that is probably sufficient for 32P labeling of hemoglobin messenger RNAs (mRNA) in quantities commensurate with structural characterization of mRNAs by radioautographic methods.

In vitro cultures of cloned murine erythroleukemic cells, chronically infected with Friend leukemia virus, form material that stains with benzidine. This material has the spectral, iron incorporative, peroxidatic, and electrophoretic properties of hemoglobin. Growth in medium containing dimethylsulfoxide (DMSO) results in erythroid differentiation of nearly 100%
of leukemic cells and profoundly augments their production of hemoglobin. These findings not only offer an opportunity to study early stages of erythropoieticlike differentiation in a manner heretofore impractical, but also provide a much needed model setting for detailed structural analyses of hemoglobin messenger RNAs (mRNA). Prerequisites to such analyses are exact delineation of the hemoglobin polypeptides produced and demonstration that cellular synthetic capacity is sufficient for high specific activity $^{32}$P labeling of mRNAs in quantities communnsurate with structural characterization by the radioautographic methods of Sanger et al.4

In this report we establish: (1) that mouse adult $\alpha$- and diffuse-type $\beta$-chains and peptides, typical of hemoglobin in the DBA/2J strain from which leukemic cells were derived 6 yr earlier, are produced by DMSO-treated cultures; and (2) that the rate of hemoglobin production in such cultures reflects a synthetic capacity adequate for mRNA characterization.

**MATERIALS AND METHODS**

**Tissue Culture and Preparation of Radiolabeled Protein**

Origin and tissue culture of murine, Friend virus-induced leukemic cells are detailed elsewhere.1-3 In the present experiments, mouse leukemic cells were seeded $10^5$/ml and grown as 10-mi aliquots at 37°C in dehydrated basal medium (Eagle) diluted with Earle's balanced salt solution (Grand Island Biological). This medium was supplemented with 15% (v/v) unheated fetal calf serum (Grand Island Biological) containing a measured 400 $\mu$M/liter lysine. Final lysine concentration in mixture was 228 $\mu$M/liter; final leucine concentration was approximately 200 $\mu$M/liter. Two per cent (w/v) dimethylsulfoxide (DMSO) was added from the start to all but control incubations.3

After 72-hr growth (control incubation No. 1 and DMSO-treated incubation No. 2) or 78-hr growth (DMSO-treated incubations No. 3 and 4) in the medium just described, cells were removed to a medium that was similar, except that fetal calf serum and 40 $\mu$Ci $^{14}$C-L-lysine (supplied as 312 $\mu$Ci/µM by New England Nuclear) now constituted the sole sources of lysine. Final lysine concentration then became 74 $\mu$M/liter, while specific activity was 54 $\mu$Ci/µM. Sixteen hours later (incubations No. 1 and 2) or 17 hr later (incubations No. 3 and 4), $2 \times 10^7$ cells were harvested by centrifugation.

Incubations No. 5, 6, and 7 differed in that (1) approximately 500 $\mu$Ci $^{3}$H-DL-leucine (5000 $\mu$Ci/µM, Nuclear, Chicago, Ill.) was used instead of radioactive lysine, (2) radioisotope was added at the time of seeding, (3) no attempt was made to limit carrier amino acid concentration, and (4) duration was 96 hr following which cells were recovered by centrifugation. Incubations No. 5 and 7 were treated with DMSO, while No. 6 was not.

Incubations No. 1–4 utilized cells from leukemia cell clone 745, whereas incubations No. 5–7 derived from clone 707.

Following harvest, cells of all incubations were washed twice with large volumes of 0.14 M NaCl, lysed in 0.5 or 1.0 ml of 1 mM phosphate pH 7.4 buffer, and further disrupted by freezing-thawing through several complete cycles (four cycles for incubations No. 1–2, two for No. 3, seven for No. 4, and two for No. 5–7). Radioassay5 before and after dialysis of lysate indicated that almost all unincorporated radioisotope was removed during cell washing.

After cell lysis, supernatant and stromal fractions from incubations No. 1–4 were separated by 15,000 rpm centrifugation in a Beckman model 152 Microfuge for 5 min at 4°C, and thereafter, each of the fractions was processed separately.

**Immunoprecipitation of Hemoglobin**

Immunoprecipitation of hemoglobin from leukemic cell culture lysates, in the presence of a much larger quantity of radiolabeled5 authentic marker hemoglobin from DBA/2J
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mouse reticulocytes, was used as a first step in characterization and quantitation. Four-tenths to 0.9 ml aliquots of 14C-lysine-labeled protein, representing known portions of 15,000 rpm supernatant fluid from lysates of leukemic culture incubations No. 1–4, were mixed with small volumes of reticulocyte lysate containing approximately 50 μg hemoglobin and known amounts of 3H-lysine-labeled protein. Each such antigen mixture was then reacted for 48 hr at 4°C with 1.5 ml of rabbit antiserum prepared6 against hemoglobin A from the New World spider monkey (Ateles)7 but strongly cross-reactive with DBA/2J mouse hemoglobin on immunodiffusion plates. The proportions of antiserum and authentic mouse 3H-hemoglobin antigen in reaction mixtures were sufficient, in preliminary analysis, to precipitate (1) 85–95% of hemoglobin after 48 hr at 4°C, as judged by quantities of radioactivity remaining in supernatant before washing; and (2) 40–70%, as judged by radioactivity remaining in precipitates after three to four washes with 0.14 M NaCl.

Estimation of Hemoglobin Synthesis in Leukemic Cell Cultures

Washed immunoprecipitates were completely dissolved in known volumes of the pH 6.7 buffer, as described by Clegg et al.8 Small aliquots of dissolved precipitate were assayed for radioactivity in Bray's solution containing 2.5% (w/v) thixotropic gel powder (Packard). In incubations No. 1–4, the quantity, C, of 14C-labeled globin initially present (before losses) in supernatant fluids of leukemic cell culture lysates was estimated, as illustrated in Table 1, using the expression T/R = C, where T is μCi of authentic 3H-hemoglobin added before immunoprecipitation and R is the 3H/14C (dpm/dpm) ratio in dissolved precipitate. Similar estimates were later obtained using hemoglobin derivatives, such as polypeptide chains and tryptic peptides. As will emerge, final estimates of C did not depend on exclusion of nonhemoglobin from immunoprecipitates, but rather relied on values of R obtained from extensively purified tryptic peptides (Table 3).

Chromatography of Hemoglobin Chains

Each cell lysate was subject to chain separatory chromatography on carboxymethyl (CM) cellulose (Whatman CM-32) columns.8 In the case of incubations No. 1–4, this included chromatography of dissolved 3H- and 14C-labeled protein from each immunoprecipitate and—separately—chromatography of 14C-labeled leukemic cell lysate stroma after 75–100% dissolution of radioactive protein in the urea-mercaptoethanol buffer. Unfractionated, but stroma-free, mouse hemolysate was added as carrier to all samples prior to chromatography. In addition, known quantities of 3H-labeled mouse hemoglobin were added as markers to the 14C-labeled leukemic cell stroma. Processing of 3H-labeled incubations No. 5 and 6 differed in that globin from the entire lysate was precipitated with acid-acetone10 in the presence of carrier and 14C-labeled mouse hemolysate. Globin was thereafter fractionated by CM-cellulose chromatography. Processing of 3H-labeled supernatant fluid from DMSO-treated incubation No. 7 followed the procedure previously described by Scher et al.2 Fractions from all CM-cellulose columns were analyzed by both radioassay5 and absorption at 280 nm.

Characterization of Tryptic Peptides from Isolated Hemoglobin α- and β-Chains

Peak fractions containing carrier α- and β-chains from all but one of the eight CM-cellulose columns used for processing incubations No. 1–4 were isolated, and S-aminoethylcysteinyl (AE) derivatives were prepared.8,11 The exception was the immunoprecipitated fraction of incubation No. 1 that, in the hemoglobin chain peak fractions, contained too little radioactivity for further analysis. Each of the seven α- and seven β-chain samples was individually digested with trypsin.12 After digestion, insoluble residue was removed and not analyzed further. Soluble tryptic peptides from each digestion were separated by column chromatography on Dowex 50 as described by Jones.11 Following radioassay of Dowex-50 column fractions, α- and β-chain peptide peaks from the immunoprecipitate fractions of incubations No. 2–4 and stromal fractions of incubations No. 1–4
were further purified by preparative paper electrophoresis at pH 4.7. A portion of each peptide was assayed for radioactivity. Another portion was hydrolyzed with 5.7 N HCl, and proportions of amino acids were estimated. Analyses were of a quality illustrated elsewhere. Results were compared with known compositions of mouse hemoglobin peptides, and, in this manner, we were able to identify each of the soluble, lysine-labeled, tryptic peptides present in α- and diffuse-type β-chains. The peptides thus characterized were identical to those reported for the AKR mouse strain.

**Microscopic Single-Cell Radial Immunodiffusion Assay**

Freshly obtained peripheral blood cells from nonanemic mice and cells from leukemic cell cultures were analyzed by a modification of the microscopic immunodiffusion assay developed by Gitlin et al., and, independently, by Daufi and Rondell. Leukemic cell cultures used were exactly comparable to those employed in incubations No. 1–4, except they contained 228 μM nonradioactive lysine and were incubated for 100 hr after seeding. Both 2% DMSO-treated and untreated cultures were examined. Preparations for single-cell immunodiffusion assay were obtained by mixing, at 40°C, approximately 0.2 ml of 0.5% agarose (Bio-Rad) containing 0.14 M NaCl with 1/10 vol of washed cells to a final concentration of 10^5–10^6/μl. Ten to 30 μl drops of these mixtures were individually placed on warmed microscope slides, gently but quickly capped with cover slips, and promptly chilled to 4°C for a few minutes. Considerable care was required in handling cell-agar mixtures, since mouse cells (in contrast to human cells) are particularly susceptible to shear lysis during cover slip application. After a few minutes, cover slips were removed and the approximately 50-μ-thick agar-cell films were briefly reacted in a humid chamber with 10–50-μl overlays of rabbit serum containing 50 μg/ml gramicidin (Nutritional Biochemical). Gramicidin, diluted with serum from a stock solution of 5 mg/ml in propylene glycol, served as a delayed lytic agent. Cover slips were reapplied within a few minutes, and slides were held in a humid chamber until examined in a microscopic dark field at approximately 100–1000X magnifications. Cell lysis began within a short time and was complete for all cells within about 18 hr. In many instances, the entire course of immunoprecipitate plaque formation around an individual cell could be observed during a 15–60-sec interval. Cells from all sources were reacted with several different dilutions of the rabbit antihemoglobin described earlier, as well as with serum from a nonimmunized rabbit. Immunoprecipitate plaque diameters were measured for at least 200 cells. Standardization of the relationship between plaque diameter and hemoglobin concentration was obtained, following the method of Mancini et al., described by Gitlin et al., using peripheral blood erythrocytes from individual mice whose mean corpuscular hemoglobin had been calculated. After extensive measurement of immunoprecipitate plaque diameters, cover slips were removed from all preparations; gel slices were washed in petri dishes through large volumes of 0.14 M NaCl, reacted with fluorescein-conjugated sheen antirabbit IgG (Miles Laboratories), again washed, remounted, and examined in a fluorescent dark field with a Zeiss Ultraphot III microscope. Although there was no significant change in either the mean or statistical distribution of immunoprecipitate plaque diameters, photography of leukemia cells was now made easier because of enhanced contrast between fluorescent immunoprecipitates and sometimes overlying intracellular granules.

**RESULTS AND DISCUSSION**

**Identification and Characterization of Hemoglobin Synthesized by Leukemic Cell Cultures**

As shown in the left panel of Fig. 1, much of the immunoprecipitated protein from a DMSO-treated culture cochromatographs with authentic ^3H-labeled and carrier adult mouse hemoglobin α- and β-chains. This was not true of the control culture (right panel, Fig. 1) that was untreated with
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DMSO and where most of the immunoprecipitated $^{14}$C material appears unrelated to $\alpha$- and $\beta$-chains. The lack of $^{14}$C counts in the main portion of the control chromatogram (right panel, Fig. 1) incidentally suggests that the minor $^{14}$C peaks present in the "pre-$\beta$" region of the DMSO-treated preparation (left panel, Fig. 1) are related, in some unknown manner, to hemoglobin rather than simply nonspecifically trapped substances. Similar "prepeak" phenomena have been observed during fractionation of hemoglobin from other sources.17-18

Fig. 1. Chromatography of dissolved immunoprecipitates (from hemoglobin-antihemoglobin reaction) on CM-cellulose columns developed at pH 6.7 with a salt gradient containing 8 M urea and 0.05 M mercaptoethanol.8 Prior to application, each dissolved immunoprecipitate was augmented with unfractionated hemolysate from DBA/2J adult mice. Left panel, sample contained immunoprecipitated $^3$H-lysine-labeled protein from DBA/2J adult mouse reticulocytes and $^{14}$C-lysine protein from supernatant fraction of 2% DMSO-treated leukemic cell culture (incubation No. 2). Right panel, sample was similar except $^{14}$C material in immunoprecipitate came from supernatant of a control leukemic cell culture not treated with DMSO (incubation No. 1) but otherwise identical to incubation No. 2. After elution, about one-seventh of each fraction was assayed for radioactivity as described in text. The falling 280 nm absorbance baseline is characteristic of samples applied as hemoglobin.
The results shown in Fig. 1, left panel, for incubation No. 2 were corroborated by analysis of incubations No. 3, 4, and 7. In the latter case, purification by immunoprecipitation was omitted, and three hemoglobin components were isolated by a combination of ammonium sulfate fractionation and electrophoresis. Each component when isolated, converted into globin, and analyzed by CM-cellulose chromatography exhibited \( \alpha \) and \( \beta \)-chain peaks similar to those shown in Fig. 1, left panel. Thus, although the origin of differences between the three electrophoretically distinguishable components is unclear, these differences are not manifest at the level of chain separation.

The nature of the chain-related material from DMSO-treated cultures was firmly established by comparing the chromatographic and subsequent electrophoretic behavior of tryptic peptides with \(^3\)H-labeled peptides from authentic, adult mouse hemoglobin. In Fig. 2, there is point-for-point concordance between \(^3\)H and \(^{14}\)C among hemoglobin \( \alpha \) and \( \beta \)-tryptic peptides, including peptide \( \beta \)-2b (residues 14–17) characteristic of diffuse-type mouse hemoglobin in aminoethylated preparations. This is not to say that the hemoglobin synthesized by leukemic cell cultures is identical in every respect to that found in adult DBA/2J mice. Our analysis was confined to soluble tryptic peptides labeled with lysine. Insoluble peptides and those not labeled with lysine were not examined. The aggregate of these unanalyzed peptides contains

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**Fig. 2.** Column chromatography on Dowex 50 resin of soluble tryptic peptides prepared from S-aminoethyl-cysteinyl derivatives of \( \alpha \) (upper panel) and \( \beta \) (lower panel) hemoglobin chains isolated from peak fractions (\( \alpha \): fractions 40–45; \( \beta \): fractions 32–36) shown in left panel of Fig. 1. All \(^{14}\)C-labeled materials derive originally from supernatant proteins in incubation No. 2 leukemic cell culture lysate. After elution, about one-seventh of each Dowex 50 fraction was assayed for radioactivity.
Table 1. Immunoprecipitation of Mixtures of $^3$H-Lysine Hemoglobin From Mouse Reticulocytes and $^{14}$C-Lysine Protein From Mouse Leukemia Cell Cultures: Illustration of Data and Calculations*

<table>
<thead>
<tr>
<th>Incorporation</th>
<th>Incubation No. 1 (no DMSO) ($\mu$Ci)</th>
<th>Incubation No. 2 (DMSO) ($\mu$Ci)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble $^{14}$C-protein initially present</td>
<td>4.18</td>
<td>8.76</td>
</tr>
<tr>
<td>$^3$H-Hemoglobin initially added $= T$</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>$^3$H-Hemoglobin recovered in immunoprecipitate</td>
<td>0.37</td>
<td>0.35</td>
</tr>
<tr>
<td>$^3$H/$^{14}$C Ratio in immunoprecipitate $= R$</td>
<td>2.50</td>
<td>0.73</td>
</tr>
<tr>
<td>Calculated $^{14}$C &quot;hemoglobin&quot; initially present $= C = T/R$</td>
<td>0.30</td>
<td>1.03</td>
</tr>
</tbody>
</table>

*Calculations used here and in Tables 2-4 are described in Materials and Methods.

88 out of 145 $\beta$-residues and 88 out of 141 $\alpha$-residues. Therefore, we analyzed peptides with the minority of residues.

The quantity of $^{14}$C-labeled hemoglobin chains synthesized by leukemic cultures not treated with DMSO is, as expected, substantially less than in treated preparations and in the case of incubation No. 1 (right panel, Fig. 1) is too small for definitive characterization. Nonetheless, a pattern of $\alpha$- and $\beta$-chain chromatography in an untreated incubation (No. 5) was obtained when (1) the quantity of radioactive protein synthesized was much increased through the use of 500 $\mu$Ci of $^3$H-leucine and (2) the entire leukemic cell lysate, including stroma, was converted into globin and applied to columns. In both incubations No. 5 and 6, baseline noise from nonhemoglobin proteins was substantially less than that seen with stromal preparations. We attribute the seeming purity of these preparations to the globin preparation step.

Quantitation of Radioactive Hemoglobin Synthesized by Leukemic Cell Cultures and the Disparity Between Amounts in Supernatant and Stromal Fractions

The radioactivity in immunoprecipitable hemoglobin, i.e., the supernatant fraction of leukemic cell lysates, forms a disappointingly small proportion of the 40 $\mu$Ci $^{14}$C-lysine initially added to incubations No. 1-4. Moreover, estimates of the proportion incorporated into hemoglobin further declined with successive peptide purification (Table 2) and became relatively constant only in the last steps (Table 3).

The paucity of hemoglobin in all immunoprecipitates (Table 4), when taken together with occurrence of light red to brown stroma and nearly colorless supernatants in much of the DMSO-treated leukemic cell lysates, strongly suggested that the bulk of hemoglobin was insoluble. Estimates of stromal hemoglobin bear this out. Except for the omission of immunoprecipitation, such estimates of $^{14}$C-labeled stromal hemoglobin were obtained in the same manner as for supernatant hemoglobin. Although the $^{14}$C background counts remained higher in stromal preparations than in immunoprecipitate derivatives, the chromatographic behavior (not shown) of chain and tryptic peptide peaks nonetheless generally corresponded to those from supernatant fraction. In
the last steps of isolation, $^{3}$H/$^{14}$C ratios became identical for many peptides. Estimates of stromal hemoglobin obtained from purified peptides (Table 4) considerably exceed, with the possible exception of $\alpha$-chain from non-DMSO-treated incubation No. 1, the quantities in supernatant hemoglobin.

The proportion of hemoglobin between supernatant and stromal fractions (Table 4) is quite unlike that found in lysates of adult DBA/2J mouse reticulocytes, where in unpublished studies less than 2% of incorporated label is found to be associated with stroma from frozen-thawed lysates of low concentrations of cells. It seems likely that the large proportion of stromal hemoglobin found in leukemic cell lysates is due to denaturation. Immuno-diffusion estimates of hemoglobin content per cell, discussed in a subsequent section, are consistent with this interpretation. Although quantities of hemoglobin per cell thus measured vastly exceed estimates based on radiolabel incorporation into soluble supernatant hemoglobin, the total quantities of hemoglobin per cell are found to be nearly the same by either method. This suggests that the relative abundance of stromal hemoglobin, as shown in Table 4, is not due to some intrinsic property of leukemic cells, but rather is the result of denaturation during hypotonic and freeze-thaw lysis. This is perhaps expected, since mouse diffuse-type hemoglobin is capricious in its stability and often precipitates when thawed after frozen storage.

The data in Table 4 bear on the question of whether mouse hemoglobin chains, other than $\alpha$ and diffuse-type $\beta$, are synthesized by mouse leukemic cell cultures. Although the existence of unidentified chains cannot be rigorously excluded, the lack of any consistent excess of either $\alpha$- or $\beta$-chain synthesis in Table 4 suggests that the quantities of other chains, if present, are small. Such reasoning presupposes that at least one kind of unidentified chain would be associated, as in man, with either $\alpha$ or $\beta$. The lack of consistent excess of either $\alpha$- or $\beta$-chain synthesis also suggests that the production of these two chains is relatively balanced during early stages of erythropoiesis in culture.

Total picograms (pg) of hemoglobin produced per synthetically active cell can be calculated from data in Table 4. Assuming from single-cell immuno-diffusion assay that 88% of cells in radiosynthetic incubations are producing hemoglobin, the sums of supernatant plus stromal hemoglobin given in

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>$\alpha$-Chain ((\mu)Ci)</th>
<th>$\beta$-Chain ((\mu)Ci)</th>
<th>Total ((\mu)Ci)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoprecipitate</td>
<td>0.23</td>
<td>0.19</td>
<td>0.42</td>
</tr>
<tr>
<td>CM-cellulose peak fractions</td>
<td>0.16</td>
<td>0.14</td>
<td>0.30</td>
</tr>
<tr>
<td>Dowex-50 peptide</td>
<td>0.16</td>
<td>0.12</td>
<td>0.28</td>
</tr>
<tr>
<td>pH 4.7 electrophoresis peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Estimates use the expression, $C = T/R$, illustrated in Table 1 and discussed in text. **Estimates based on mean values shown in Table 3.
Table 3. Tritium/\(^{14}\)C (dpm/dpm) Ratios in Isolated Tryptic Peptides Derived—from Chain Separation—from Immunoprecipitates of \(^3\)H-Lysine-Labeled Mouse Hemoglobin and \(^{14}\)C-Lysine-Labeled Mouse Leukemia Cell Incubation No. 2: Results Before and After Preparative Electrophoresis

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>(\alpha)-Chain Before Electrophoresis</th>
<th>(\alpha)-Chain After Electrophoresis</th>
<th>(\beta)-Chain Before Electrophoresis</th>
<th>(\beta)-Chain After Electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.29</td>
<td>2.25</td>
<td>1</td>
<td>2.83</td>
</tr>
<tr>
<td>2</td>
<td>2.26</td>
<td>2.33</td>
<td>2b</td>
<td>2.92</td>
</tr>
<tr>
<td>3</td>
<td>2.19</td>
<td>2.29</td>
<td>6</td>
<td>2.79</td>
</tr>
<tr>
<td>5*</td>
<td>2.34</td>
<td>2.74</td>
<td>7</td>
<td>3.13</td>
</tr>
<tr>
<td>6*</td>
<td>2.24</td>
<td>ND(a)</td>
<td>8</td>
<td>2.34</td>
</tr>
<tr>
<td>7</td>
<td>2.52</td>
<td>2.43</td>
<td>10</td>
<td>2.50</td>
</tr>
<tr>
<td>Mean</td>
<td>2.27</td>
<td>2.33</td>
<td></td>
<td>2.71</td>
</tr>
</tbody>
</table>

*Bracketed peptides are not resolved during Dowex-50 chromatography, i.e., before electrophoresis.
\(a\)Not determined.

Table 4 represent for incubations No. 2–4, respectively, production of 1.19, 4.53, and 3.49 pg hemoglobin/synthetically active cell, during 16–17-hr periods. The mean of the two higher estimates, approximately 4 pg/active cell, corresponds to about one-fourth of the 15 pg present in an individual adult mouse erythrocyte.

**Quantitation of Hemoglobin Produced by Leukemic Cell Cultures—Estimation by Immunodiffusion**

A quantitative estimate similar to that just given can be obtained independently by microscopic immunodiffusion assay of hemoglobin contained in single cells.\(^{13,14}\) Errors in estimating the capacity of hemoglobin synthesis by leukemic cells, which essential for gauging the quantities of mRNAs present, are thereby precluded. Examples of immunodiffusion assay are given in Fig. 3. As illustrated in Fig. 3A and C, hemoglobin from all adult DBA/2J mouse erythrocytes and 88% of DMSO-treated leukemic cells reacted, after lysis with gramicidin,\(^{15}\) with rabbit antihemoglobin to form plaques of granular immunoprecipitates. No reaction attends normal rabbit serum (Fig. 3B). The quantity\(^{16}\) of hemoglobin in leukemic cells, incubated for 100 hr with 2% DMSO under conditions similar to those given in Table 4, ranged among 291 cells from 1.5 to 57 pg/active cell, mean = 8.5. This value is more than twice the approximately 4 pg/active cell previously calculated for the two higher radiosynthetic assays. Disparity between kinds of estimates is not surprising since assay with radiolysine was limited to 78–95-hr intervals after starting DMSO treatment, whereas immunodiffusion assay encompassed all of the hemoglobin produced during 100 hr incubation. Prior studies of rates of \(^{59}\)Fe incorporation (Fig. 3 in Ref. 3) suggest that two-thirds, i.e., 5.7 pg/active cell, of the hemoglobin measured by immunodiffusion was produced after the first 72 hr of DMSO treatment. These 5.7 pg hemoglobin/active cell
Table 4. 14C-Lysine Incorporation Into Supernatant and Stromal Fractions of DMSO-treated Leukemic Cell Lysates*

<table>
<thead>
<tr>
<th></th>
<th>Incubation No. 1:</th>
<th>Incubation No. 2:</th>
<th>Incubation No. 3:</th>
<th>Incubation No. 4:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No DMSO, 72-88-Hr</td>
<td>DMSO-treated, 72-88-Hr</td>
<td>DMSO-treated, 78-95-Hr</td>
<td>DMSO-treated, 78-95-Hr</td>
</tr>
<tr>
<td></td>
<td>14C Incubation</td>
<td>14C Incubation</td>
<td>14C Incubation</td>
<td>14C Incubation</td>
</tr>
<tr>
<td>Total Protein</td>
<td>β</td>
<td>α</td>
<td>β</td>
<td>α</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.024</td>
<td>0.036</td>
<td>0.12</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>8.76</td>
<td>0.12</td>
<td>0.16</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>0.10</td>
<td>0.06</td>
<td>5.78</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.06</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>Supematant fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal fraction</td>
<td>3.32</td>
<td>0.042</td>
<td>0.033</td>
<td>11.39</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>0.25</td>
<td>24.06</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>1.25</td>
<td>14.65</td>
<td>1.12</td>
</tr>
<tr>
<td>Sum</td>
<td>7.50</td>
<td>0.066</td>
<td>0.069</td>
<td>20.15</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>0.41</td>
<td>25.26</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>1.31</td>
<td>1.31</td>
<td>20.43</td>
<td>1.24</td>
</tr>
<tr>
<td>Sum β + α hemoglobin</td>
<td>0.135†</td>
<td>0.76</td>
<td>2.89</td>
<td>2.23</td>
</tr>
</tbody>
</table>

*Calculations are based on method illustrated in Table 1 and, except as noted, use 3H/14C ratios derived in each case from mean values among purified tryptic peptides, e.g., Table 3.

†Estimates in supernatant fraction from incubation No. 1 are based only on assay of CM-cellulose column peaks. These estimates, in light of results shown in Table 2, are undoubtedly excessive.
were thus produced in the 28-hr interval between 72 and 100 hr. In the estimates using radiolysine, about 4 pg hemoglobin/active cell were produced in the 17-hr interval between 78 and 95 hr. The rates of hemoglobin synthesis measured by the two kinds of assay are essentially the same.

Fig. 3. Microscopic dark-field photographs (× 500) of radial immunodiffusion assay of hemoglobin from individual erythroid cells.13,14 (A). Reaction is between rabbit anti-hemoglobin and adult DBA/2J mouse erythrocytes. (B). Reaction is between normal rabbit serum and virus-infected murine leukemic cells grown for 100 hr in medium containing 2% DMSO. (C). Reaction is between rabbit anti-hemoglobin and the leukemic cells just described. In A and C, immunoprecipitate plaques have been photographed in fluorescent dark field following reaction, as described in text, with fluorescein-conjugated sheep anti-rabbit IgG. This maneuver improved contrast between immunoprecipitates and sometimes overlying intracellular granules. Unreactive cells (B) are shown in conventional dark field, since they are nearly invisible in fluorescent dark field.
Speculation: The Utility of Leukemic Cell Cultures for Characterization of Globin mRNA

The estimate of about 4 pg of tetrameric hemoglobin/active cell corresponds to the production of approximately $7.2 \times 10^7$ molecules of each kind of globin chain. Given an average number of five ribosomes per active mRNA template and a chain translation time of about 20 sec at $37^\circ$C, then approximately 15 polypeptide chains will be translated per minute from each mRNA molecule. Thus, for each kind of polypeptide chain, the participation of at least $4700$ mRNA molecules/cell, or a total of $8.5 \times 10^{10}$ mRNA molecules among the $1.8 \times 10^7$ active cells present, would be required to produce amounts of hemoglobin chains found after 17-hr incubation at $37^\circ$C. Moreover, $8.5 \times 10^{10}$ mRNA molecules, each about 200,000 daltons in size, represent about 0.03 µg. Combined, the α- and β-mRNA populations form 0.06 µg. If, without compromising cellular viability, mRNA molecules can be sufficiently labeled with high specific activity $^{32}$P during the early days of DMSO treatment, then after 70–90 hr the quantity present in incubations with 16 times the number of cells used here should closely approximate the minimal amounts of 0.5 µg $^{32}$P-mRNA required for radioautographic structural characterization by Sanger’s methods. Success will depend on the development of procedures entailing minimal losses, whereby hemoglobin mRNAs (responsible for only about one-ninth of the total protein synthesis shown in Table 4) can be separated from other mRNAs. Recent isolation of ovalbumin-synthesizing polysomes by Palacios et al. suggests that it may, indeed, become possible to “fish out” single kinds of mRNA from complex mixtures such as those present in leukemic cells.

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Hemoglobin Biosynthesis in Murine Virus-induced Leukemic Cells In Vitro: Structure and Amounts of Globin Chains Produced

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