Accumulation of HbF and HbA in individual erythroid clones, or bursts, grown in culture from early human erythroid progenitor cells (BFUe, erythroid burst-forming unit) was measured using sensitive and specific radioimmunoassays. HbF and HbA could be quantitated simultaneously in individual bursts and total hemoglobin levels as low as several hundred picograms per burst could be measured. Bursts from fetal liver, umbilical cord blood, and adult peripheral blood were studied under comparable culture conditions in order to facilitate quantitative comparison of the amounts of HbF and HbA they had produced.

Individual bursts grown from fetal liver contained an average of 95% HbF (S.D. = 5.3) by the 15th culture day and demonstrated considerable homogeneity in that all bursts contained high percentages of HbF and correspondingly little or no HbA. Bursts grown from two different umbilical cord blood samples contained an average of 69% HbF and 30% HbF by the 14th culture day. Bursts from both samples demonstrated great heterogeneity (S.D. = 25.3 and 21.0, respectively) with respect to the percentages of HbF they had synthesized. Bursts of adult origin harvested on the 15th culture day contained an average of 25% HbF while bursts harvested on the 18th culture day contained an average of 13% HbF. Bursts studied at 18 days of culture demonstrated considerably more homogeneity (S.D. = 12.8) with respect to the range of percentages of HbF they had synthesized when compared to bursts harvested on day 15 (S.D. = 19.9). Most bursts of adult origin had accumulated high percentages of HbA and corresponding low amounts of HbF.

The relative production of HbF and HbA by progenitors from different sources was appropriate for the developmental age of the source; thus, this characteristic appears to be determined in vivo. Bursts grown from different sources varied in the diversity of percentages of HbF they synthesized. Umbilical cord progenitors, present during the transition from one developmental stage to another when HbF is being replaced by HbA, yielded bursts with the greatest diversity with respect to the percentages of HbF they synthesized.

Populations of bursts of adult origin maturing at later times in culture appeared to synthesize a smaller proportion of HbF than bursts which had become mature earlier.

The sequential appearance of different hemoglobins during ontogeny in various mammalian species, including man, is a striking example of the regulated expression of specific genes during cellular differentiation. In man, a major switch of hemoglobin phenotype occurs in the perinatal period during which fetal hemoglobin (HbF, 1, a2y2), the predominant hemoglobin of intrauterine life, is almost completely replaced by adult hemoglobin (HbA, 2α2). Recently, it has become possible to approach questions concerning the differential expression of the γ- and β-globin genes by hemoglobin-synthesizing cells in vitro. Tissue culture techniques are available which permit the clonal growth of early human erythroid stem cells, termed BFUe, or erythroid burst-forming units, which are bipotent with respect to production of HbA and HbF (1-4). Colonies of single cell origin which undergo erythroid differentiation and produce hemoglobin can be grown in semi-solid media from precursor cells present in normal peripheral blood as well as from erythroid tissues such as bone marrow and fetal liver (5-7).

Studies of the expression of human globin genes by cultured erythroid cells have been performed using fluorescent antibody probes (4, 5, 8), globin biosynthesis (4, 5, 7-16), and cDNA/mRNA hybridization (15, 17). Such studies have provided information on the relative expression of γ- and β-globin genes by erythroid cultures from different sources at particular times after plating. We have developed sensitive and specific radioimmunoassays for HbA and HbF which can be used to detect these hemoglobins in individual colonies, or bursts, of erythroid cells grown in culture. The radioimmunoassays permit quantitation of the amounts of both these hemoglobins in the same individual burst. In experiments where the number of cells per burst was also measured we have been able to obtain an estimate of the amount of hemoglobin contained in single cultured cells.

We report here studies on the accumulation of HbA and HbF by individual bursts grown in semi-solid culture from erythroid precursors present in human fetal, neonatal, and adult tissues. We find the relative expression of γ- and β-globin genes by these bursts in culture is characteristic of the
developmental stage of the source of cells. In addition, individual bursts from sources at different developmental stages display significant variation in the frequency of percentages of HbF they synthesize. Furthermore, studies of the immunization of HbF and HbA as a function of time in culture suggest that there is a difference in the relative expression of $\alpha$- and $\beta$-globin genes by bursts which become mature at different times after initiation of culture.

**Materials and Methods**

**Hemoglobin Purification**—Hemoglobins were isolated from erythrocytes of normal volunteers, or pooled umbilical cord blood samples by hypotonic lysis in 3 volumes of distilled water containing 1 mg EDTA, and chromatographed on DEAE-Sephadex (18). The purity of chromatographed hemoglobins was determined by isoelectric focusing between pH 7 and pH 9 in polyacrylamide gels (19). Hemoglobins were frozen as pellets in liquid nitrogen and stored at -70°C.

**Preparation of $^3H$-Hemoglobin**—Hemoglobins A and F were labeled with sodium [H]$^3$H]borohydride (Amersham, specific activity 62 Ci/mmol) by reductive methylation (20). To 2 mg of carboxy-Hb in 200 µl of water (pH 9), 0.2 µl of [H]$^3$H]borohydride (25 mCi/ml) was added. The mixture was incubated at 37°C for 10 min in a tightly capped reaction vessel in a water bath. After the reaction, the mixture was applied to a disposable PD-10 prepacked column of Sephadex G-25 (Pharmacia) equilibrated with PBS. Labeled Hb was dialyzed against B-TBS at 4°C, diluted, frozen as pellets in liquid nitrogen, and stored at -70°C. One pellet could conveniently be removed each time an assay was performed.

**Immunoabsorbant Columns**—Sephadex 1276 (NIH Animal Farm) was immunized at multiple intradermal sites with 1 ml of electrophoretically pure HbF (1 mg/ml in 0.15 M NaCl) emulsified in 1 ml of complete Freund's Adjuvant (Difco Laboratories). Two further immunoabsorptions were performed 2 weeks after the initial immunization with the same amount of antigen, and the supernatant liquid of each immunoabsorbant column was collected and stored at -70°C. The columns were washed extensively with PBS equilibrated with CO, 100 ml of 1% ethanolamine in PBS, and PBS again. In a typical experiment, a small aliquot (1-5 ml) of an antibody preparation was applied to a column, eluted with PBS, and tested in a radioimmunoassay for loss of cross-reactivity with the hemoglobin which was bound to the immunoabsorbant. If cross-reactivity had been completely removed, similar aliquots were applied to the same column, eluted, and tested until the ability of the column to absorb antibodies had been exceeded. In general, for a 5-6 ml immunoabsorbant column, this occurred after the application of between 3 and 10 ml of an ammonium sulfate-fractionated antibody preparation. Immunoabsorbant columns were stored at -70°C in the testing buffer (100 mM NaCl, 10 mM NaH2PO4, pH 7.2) and were not stripped and reused after saturation. The hemoglobin-Sepharose conjugates were stable for several months when stored in the presence of CO.

**Source and Preparation of Cells**—Fetal liver samples were obtained from aborted human embryos and were processed for culture within 6 h after collection. Gestational age was determined from the estimated fertilization date. The studies of fetal liver samples were done with procedures approved by the Human Experimentation Committee (University of Washington) after prior maternal consent.

Fetal liver pieces were obtained from several term abortions (President Laboratories, Rockville, Md.) supplemented with penicillin (100 units/ml) and streptomycin (10 µg/ml), and 5% fetal calf serum (Flow Laboratories, Rockville, Md.). Fetal liver cells were distributed to 50 ml Falcon tissue culture flasks (Falcon) for removal of the majority of adherent cells. Nonadherent cells were used for culture.

**Culture Conditions**—Unfractionated cells from fetal liver and non-adherent cells from cord blood were cultured in methylcellulose semisolid media (24). Briefly, the culture medium consisted of 1.0% methylcellulose, 30% fetal calf serum, 3% human urinary erythropoietin/ml (13.7 U/ml; preparation M-14 T NLSL obtained from Blood Diseases Branch, Division of Blood Diseases and Resources, National Heart, Lung and Blood Institute). Fetal liver cells were incubated at 1.5 X 10^5 cells/ml whereas for cord blood and adult blood cultures 3 X 10^5 cells/ml were used. The type of medium, amount of erythropoietin, and inoculum size were chosen to maximize the plating efficiency for each cell source. We have no indication that the pattern of hemoglobin accumulation is affected by these minor differences in culture conditions. Cultures were incubated at 37°C in an atmosphere of high humidity and 5% CO2 in air for varying times.

Individual mature bursts were identified from culture dishes with ultrathin pipettes at various times after initiation of culture. Bursts were mature by the criterion of intense red color in all areas of the burst when observed under a dissecting microscope. Each burst was placed in the bottom of 400-µl microfuge tubes with 5 µl of distilled water and stored frozen until analysis. The total volume of each sample was measured and the number of cells/burst was determined by counting in a hemocytometer.

The number of cells/burst was determined by counting in a hemocytometer the cells from a pooled sample of representative bursts (usually about 10-50 bursts), or in some cases counting the cells in individual bursts. In either case, the cells were dispersed and washed in a small amount of PBS before counting. Cells from bursts counted individually were centrifuged and the pellet frozen for radioimmunoassay. The number of cells in these bursts was corrected for the amount used in the hemocytometer. For every burst studied, either an individual cell count or an average value was available. These were combined with the total hemoglobin dimers per burst determined by radioimmunoassay to give average values, for each burst, of pograms of Hb/cell. An overall average for cells from the different sources studied was obtained from these values.

**Radioimmunoassay**—A double antibody radioimmunoassay was developed to measure accurately the fum of the hemoglobins of interest. For the measurement of HbF, fractions of fetal liver and placental cell culture for HbF (1.3 X 10^7 M), [H]carboxy-HbF (8.1 X 10^10 M) and unlabelled HbF (between 8 X 10^11 M and 8 X 10^10 M) or unknown samples (10-20 µl) in a total volume of 100 µl of B-TBS, were incubated in 400-µl microfuge tubes (Beckman) for 1 h at room temperature. When HbA and HbF were used to prepare antibodies (specific for HbF; 1.5 X 10^5 µl) [H]HbA (6.6 X 10^10 M) and unlabelled HbA (8 X 10^12 M to 8 X 10^10 M) or unknown samples (5-10 µl) were incubated under the same conditions. Assays were performed in duplicate and for each point a pair of control tubes were run which contained, in a medium equivalent to serum and human serum, nonspecific anti-hemoglobin antibody (200 µl) were added, mixed, and the tubes incubated at 4°C for an additional 2 h. After centrifugation for 30 min in a Beckman Microfuge

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B, the supernatants containing free labeled antigen were removed and counted in 10 ml of Aquasol (New England Nuclear) in a Searle Mark III liquid scintillation counter. The pellets containing antibody-bound labeled antigen were washed once with 100 µl of cold B-TBS, dissolved in 100 µl of 40% acetic acid, and quantitatively transferred to 10 ml of Aquasol, and the radioactivity was determined as above. The variation between duplicate determinations of B/F was generally 1-2% on the linear portion of the standard displacement curve, where all measurements were performed.

Individual bursts were removed from the freezer just prior to assay and 145 µl of distilled water containing 1 mg/ml of bovine serum albumin (Pentex) was added to each tube. The samples were subjected to three cycles of rapid freezing, thawing, and mixing on a Vortex machine. They were centrifuged for 2 min in a Beckman Microfuge B and aliquots of each supernatant were analyzed in duplicate by radioimmunoassay for content of HbA and HbF. Material remaining at the bottom of the tube was resuspended and several drops were allowed to dry on a microscope slide. After staining with Wright’s stain (25), a microscopic inspection was performed for each sample to determine the completeness of lysis. Radioimmunoassay of the supernatant obtained by centrifugation of pellets resuspended in water or in B-TBS was performed in order to examine the possibility that hemoglobin had remained associated with the pellet.

RESULTS

Reductively Methylated H-Hemoglobins—HbA and HbF used for labeling with Na ['H]borohydride each migrated as a single band on isoelectric focusing gels run between pH 7 and pH 9. Both hemoglobins could routinely be labeled with a specific activity of 2-3 µCi/µg, or a substitution level of about 7 atoms of [3H]/Hb dimer. At least 97% of each labeled hemoglobin could be precipitated by an excess of its specific antibody. The [3H]-hemoglobins were stable for at least 12 months when stored as carboxyhemoglobin, in pellets, at -70°C.

Antibodies to HbA and HbF—Antibody titers of between 5 and 10 mg/ml of anti-hemoglobin antibody were commonly obtained by immunizing sheep with HbA or HbF. These unfractionated antibodies distinguished poorly between the immunogen and the nonhomologous hemoglobin as measured by radioimmunoassay. However, in quantitative precipitin analyses of both antibody preparations, the nonhomologous hemoglobin precipitated less antibody than was maximally precipitated by the immunogen, suggesting the presence of some antibody populations specific for antigenic sites on the immunogen alone. This was not true for every serum tested and for each hemoglobin several sheep antisera were screened before an appropriate source of specific antibodies was found.

It was possible by immunoabsorption to isolate antibody populations which reacted over a large concentration range only with the hemoglobin used as immunogen. Such specific antibodies typically represented approximately 3-20% of the antibodies present in the original ammonium sulfate-fractionated serum. The antibody preparations which had been subjected to immunoabsorption contained only IgG, when studied by immunoelectrophoresis, along with small amounts of serum albumin remaining after ammonium sulfate precipitation. The presence of albumin caused the determination of the molar concentration of specific antibody by optical density to be imprecise.

Radioimmunoassays—Fig. 1 shows a standard curve for displacement of [3H]HbF from anti-HbF antibodies fractionated by immunoabsorption on Sepharose-HbA. Unlabeled HbF completely displaces [3H]HbF and demonstrates an apparent affinity constant between 10^-9 and 10^-8 M^-1. Under the same conditions, HbA is not capable of displacing [3H]HbF except at the highest concentration tested. If the displacement of [3H]HbF by HbA is extrapolated to higher concentrations, the midpoint of displacement would occur at a concentration more than 4 orders of magnitude higher than for HbF. This defines the broad concentration range over which these antibodies are specific for fetal hemoglobin.

In Fig. 2, displacement of [3H]HbA from anti-HbA antibodies fractioned on an immunoabsorbant containing HbF is illustrated. An apparent affinity constant of between 10^-9 and 10^-8 M^-1 is demonstrated. HbA completely replaces [3H]HbA from the antibodies. HbF is incapable of displacing [3H]HbA at concentrations 4 orders of magnitude higher than for HbA.

We chose the method of freeze-thawing in water for lysis of cells from erythroid bursts prior to radioimmunoassay. The presence of 0.1% bovine serum albumin to decrease nonspecific adsorption of small amounts of hemoglobin to the walls of tubes did not affect the efficiency of lysis. Freeze-thawing was previously shown to give better recovery of hemoglobin than lysis in the presence of 1% FBS, and it does not involve the addition of any components which might perturb the radioimmunoassay. Total recovery of hemoglobin by this
method was difficult to ascertain with precision because of the lack of any external control. However, examination of pellets obtained after centrifugation of lysed cells showed only swollen, darkly staining nuclei or clumps of darkly staining chromatin when spread on microscope slides and stained with Wright’s stain. No shreds of pink-stained cytoplasm, visible in undisturbed cells, were seen. When individual bursts were lysed in less than 150 μl of water such fragments of cytoplasm were seen. The pellets did not appear to contain residual hemoglobin as judged by radioimmunoassay of supernatants obtained by centrifuging resuspended pellets.

Studies of Cultured Fetal Liver Progenitors—Fetal liver progenitors produce, in culture, a continuous spectrum of maturing colonies, with the development of very large, red erythroid bursts occurring after about 10 culture days. Fetal liver progenitors, in general, demonstrate a higher cloning efficiency than cord blood and adult peripheral blood progenitors, as well as a higher proliferative potential per progenitor, since they produce the largest colonies under comparable culture conditions.

In the present study, erythroid bursts were grown in the presence of 1.5 I.U./ml of erythropoietin from a 52-gestational-day fetal liver sample. The cloning efficiency of this fetal liver culture when colonies were counted on day 14 was 271 BFUe/10^6 inoculated cells. At day 15, when most of the erythroid bursts appeared red, individual bursts were lifted off the plates with a fine pipette, placed in a microfuge tube, and immediately frozen as described above. A total of 43 bursts were studied. Individual cell counts were performed on 22 of the largest bursts and gave values of 1.5-5.2 × 10^6 cells/burst. The average size of the remaining bursts which were not individually counted was 1.28 × 10^5 cells/burst, determined from a pooled sample.

Of the 43 bursts studied a single burst contained hemoglobin levels too low to detect in the radioimmunoassays. However, 14 out of the remaining 42 bursts contained no HbA or levels of HbA too low to detect. Values between 3.4 × 10^{-12} and 1.1 × 10^{-11} mol of total hemoglobin dimer per burst were observed by radioimmunoassay. Using the data concerning the number of cells/burst, an average of 7.8 pg of HbA/cell was calculated.

A histogram of the distribution of the per cent of HbF among bursts grown from erythroid precursors in fetal liver is presented in Fig. 3. There is very marked skewing toward higher percentages of HbF. Out of 42 bursts studied, 36 contained greater than 90% HbF. Mean HbF produced was 95% (S.D. = 5.3). Cultures of fetal liver progenitors appear to synthesize amounts of HbF similar to those produced in vivo by erythroblasts and reticulocytes from the same source (14).

The low levels of accumulation of HbA in the bursts we have tested are consistent with the small amount of HbA present in peripheral blood of early fetuses (26).

Studies of Cultured Umbilical Cord Progenitors—Culturing erythroid progenitors from the neonatal period show culture characteristics different from fetal liver progenitors and are more similar to adult peripheral blood progenitors in terms of lower cloning efficiency and proliferative potential. These progenitors give rise to erythroid bursts maturing sequentially in culture from day 9-10 to the end of the 3rd week in culture.

Two umbilical cord blood samples were used for the present study. They were processed and cultured in the presence of 1.5 I.U. of erythropoietin/ml as described above. The cloning efficiency of cord blood sample I was 266 BFUe/10^5 inoculated cells and of cord blood sample II was 79 BFUe/10^5 cells. Groups of bursts collected according to size in each experiment varied between 3.5 × 10^5 and 1.2 × 10^6 cells/burst as determined by hemocytometer counting of a few pooled bursts from each group.

A total of 55 bursts were studied from cultures of umbilical cord blood sample I collected on culture day 14. One of these bursts contained levels of total hemoglobin too low to detect, and in 5 bursts, HbA was below the level of detection. Values between 7.5 × 10^{-14} and 1.1 × 10^{-11} mol of total hemoglobin dimer were observed in the radioimmunoassay. The cells contained a calculated average of 13.9 pg of hemoglobin/cell.

Using umbilical cord blood sample II, cultured under the same conditions, 29 bursts were individually collected for study on day 13. Four bursts contained levels of HbF too low to detect. Values between 1.4 × 10^{-12} and 8.3 × 10^{-12} mol of hemoglobin dimer per burst were measured. Individual cells contained a calculated average of 12.3 pg of hemoglobin.

Fig. 4 shows the distribution of percentages of HbF among bursts from the two umbilical cord blood samples. Umbilical cord progenitors produced bursts with a much larger diversity of percentages of HbF content than did fetal liver progenitors. For both samples a skewed distribution of HbF content was
obtained. The mean percentage of HbF was 69% for sample I (S.D. = 25.5) and 30% for sample II (S.D. = 21.0). The degree of heterogeneity within each sample and the widely different means probably reflect the heterogeneity of cells present during the switch from primarily HbF synthesis to primarily HbA synthesis occurring during the perinatal period. The difference in the average per cent HbF in the two samples may reflect the extent to which the neonates had been switched by the time of their birth. HbF in the peripheral blood of Sample I, determined by alkali denaturation (27), was 89%, while for Sample II it was 57%. HbF content in vivo in cord blood samples has been reported to vary between 53 and 90% (28).

Studies of Cultured Adult Peripheral Blood Progenitors—Adult peripheral blood samples were processed similarly to cord blood, as described, and cultured in the presence of 2.0 I.U. of erythropoietin/ml. Circulating erythroid progenitors in the adult yield sequentially maturing populations of erythroid bursts in culture. On day 13 the cloning efficiency was 20 BFUe/10^5 inoculated cells. Populations of individual mature bursts present on culture day 15 were collected for study. The plates from which all the hemoglobinized colonies were removed, contained also a population of bursts which appeared colorless and which were left *in situ*. The plates were returned to the incubator. Three days later, at culture day 18, most, if not all, of the previously “colorless” bursts showed intense red color and these were now removed for testing. Since subsequent crops of maturing bursts were collected in the same plates after the removal of a previously mature population, there was no overlap in the populations of bursts tested. Bursts collected on day 15 contained on average 5.1 x 10^3 cells, while those collected on day 18 contained 8.2 x 10^3 cells as determined by counting pooled bursts in a hemocytometer.

A total of 50 bursts were examined after 15 culture days. Twelve of the bursts contained levels of hemoglobin too low to detect. These bursts were the smallest studied; thus, although they were red, the total hemoglobin/burst was below the level of detection of the assay. The bias introduced in our data by this result will be discussed. In addition, 6 bursts contained undetectable levels of HbF. Values between 1.8 x 10^-14 and 3.7 x 10^-12 mol of total hemoglobin dimer were observed. Mean HbF produced was 23% (S.D. = 19.9). The cells contained a calculated average of 8.9 pg of hemoglobin/cell.

At 18 culture days, 38 bursts were studied. Only a single burst contained levels of total hemoglobin too low to measure. However, 15 bursts contained undetectable levels of HbF. Bursts contained between 8.4 x 10^-14 and 7.7 x 10^-12 mol of hemoglobin dimer. Of this total an average of 13% was HbF (S.D. = 12.8). The cells contained an average of 8.0 pg of hemoglobin/cell.

Fig. 5 is a histogram of the per cent of HbF content of bursts harvested on day 15 (*upper panel*), or on day 18 (*lower panel*). A distribution skewed toward lower values of HbF content was obtained for both groups, however, much more heterogeneity was apparent among the bursts harvested on day 15. All bursts with measurable amounts of hemoglobin synthesized large amounts of HbA as would be expected for this developmental stage.

**DISCUSSION**

**Hemoglobin Switching in Vivo and in Vitro**—The gradual change in hemoglobin phenotype which takes place in the peripheral blood of an animal is referred to as hemoglobin switching. This switching appears to be mediated by the gradual replacement of erythroblasts synthesizing a particular ratio of globin mRNAs by cells synthesizing a different ratio (29). This process is most likely related to events which take place in tissues where erythroid precursors differentiate and proliferate. Considerable evidence implicates an early erythroid stem cell, at the same or an earlier stage as the cells (BFUe) which function as burst formers in culture, as the site of regulatory processes (5, 14, 16). However, it is still not clear to what extent this process is related to the sequential proliferation of more differentiated clones, or to biochemical changes which occur in all erythroid cells during proliferation.

At the present time methods are not available for the isolation of the stem cells of interest for direct examination. However, inferences can be made about these stem cells by examining their differentiated progeny in clonal culture.

**In vitro** culture of erythroid cells utilizes semisolid support media such as plasma clots and methylcellulose to study the erythropoietin-dependent growth of single precursor cells into colonies which synthesize hemoglobin and become red (1-3, 5, 24). The cells which produce colonies under these conditions appear to be of two operationally defined types (30-32). The more differentiated CFUe, erythroid colony-forming unit, is highly sensitive to erythropoietin and typically produces small (8-64 cells) erythroid colonies after about 5-7 culture days. The less differentiated BFUe, erythroid burst-forming unit, requires higher erythropoietin concentrations for growth and has a greater proliferative capacity in culture producing multicentric colonies, or bursts, of up to 5.0 x 10^4 cells between 11 and 14 culture days. The distinction between BFUe and CFUe is not precise and there probably is a continuum of erythroid stem cells which can be cultured *in vitro* beginning with the BFUe (which is presumably closely related to the pluripotent stem cell) and ending with the CFUe, which is related by fewer cell divisions to the erythroblasts which give rise to red blood cells (3). Our studies have focused on progeny arising from the BFUe end of the erythroid stem cell spectrum because these cells are close to the level(s) at which control processes operate.

**Assays for Investigating Hemoglobin Switching**—We have developed radioimmunoassays specific for both HbF and HbA. The sensitivity of these assays is in the picogram range and they are specific for a single hemoglobin species over a
wide concentration range (i.e., 4 orders of magnitude). These characteristics have enabled us to use the assays to quantitate HbF and HbA in individual erythroid bursts grown in culture from single erythroid progenitors. The amounts of other hemoglobins such as HbAz are presumably small in comparison to HbF and HbA in the cells we have studied.

Previous studies of hemoglobin in erythroid cell cultures have employed a variety of techniques. Fluorescent antibody labeling using specific anti-HbF or anti-HbA tagged with different fluorescent molecules has given qualitative information on the presence or absence of HbF in clones grown from human bone marrow and adult peripheral blood progenitors (4, 5, 8). This technique was used to establish the single cell origin of erythroid bursts in culture (5). The ratio of \( \gamma/\gamma^* \) chain synthesis at a particular time in culture has also been used as a measure of globin gene activity. Pooled erythroid clones (whole culture dishes) have been analyzed (4, 7–11, 14–16) as well as individual bursts (12, 13). Ratios of chains were established either by column separation of labeled chains on CM-cellulose, or by isoelectric focusing and fluorography.

The radioimmunoassays we have employed enabled us to measure ratios of HbA and HbF produced, as well as to quantitate the total accumulation of these hemoglobins. Quantitation by radioimmunoassay avoids the possibility of ratios being influenced by the inclusion of the pre-\( \beta \) peak under the \( \gamma \) peak of radioactivity in CM-cellulose chromatography, and the inclusion of non-globin radioactivity under the HbA or HbF position, or chain exchange, in isoelectric focusing. Radioimmunoassays specific for HbF have been reported (33, 34). One of these, of similar sensitivity to our own, has been used in the study of HbF in erythroid cell cultures (34). A radioimmunoassay for HbA has not, to our knowledge, been previously reported.

Using the radioimmunoassay we obtained, in five different experiments, average values of hemoglobin of 7.8, 8.0, 8.9, 12.3, and 13.9 pg/cell. Circulating red blood cells contain about 30 pg of hemoglobin/cell. By morphologic criteria, erythroid cells in tissue culture do not reach this final stage of differentiation, therefore one might expect values somewhat lower than 30 pg of hemoglobin/cell. In vivo levels of hemoglobin of 13 pg/cell for proerythroblasts, which these cells resemble, have been reported (35). Nevertheless, we consider our results to represent a lower limit for a number of reasons.

First, although it was attempted to choose uniformly mature colonies there is a lack of synchrony among cells in individual bursts with respect to accumulation of hemoglobin. Second, after 18 days in culture, the latest time at which the bursts we studied were collected, bursts continue to synthesize hemoglobin. Finally, it remains possible that technical problems such as incompleteness of lysis of cells prior to radioimmunoassay, and inaccuracy in counting the number of cells per burst, have contributed to the finding of low levels of hemoglobin per cell. There were 7 bursts out of 200 bursts examined which had hemoglobin levels greater than 30 pg/cell. Levels of hemoglobin in cultured erythroid cells in this range have been suggested by others.¹

¹ Th. Papayannopoulou and G. Stamatoyannopoulos, unpublished observations.

⁴ G. J. Dover and S. H. Boyer, personal communication.

² Differences between Precursors from Different Developmental Sources—In our studies, as judged by HbF accumulation of their progeny, fetal liver precursors demonstrated little heterogeneity with respect to the percentage of HbF they will synthesize. Most bursts demonstrated high levels of HbF and reciprocally low levels of HbA. These data confirm earlier qualitative immunofluorescent studies (36). The low levels of HbA found in the bursts reflects the low levels of this hemoglobin circulating in the blood at this developmental age (26). We found bursts from fetal liver precursors had accumulated an average of 95% HbF by the 14th culture day, which correlates well with the \( \gamma/(\gamma + \beta) \) ratios previously published for bursts grown from fetal liver precursors. These range between 0.80 and 0.94 in studies done after culture for 5–14 days (7, 14, 16).

In contrast to fetal liver bursts, two umbilical cord samples produced bursts with much more heterogeneity with respect to the levels of HbF they synthesized. In our studies, individual umbilical cord bursts synthesized every level between 0% and 100% HbF. Similar results have been obtained in biosynthetic studies. Ratios of \( \gamma/(\gamma + \beta) \) from 0.14 to 0.85 have been reported in studies done between the 9th and 20th culture days (6, 12, 14, 16). The increased heterogeneity is likely to reflect the diversity of precursor cells present during the perinatal period when the HbF to HbA switch is being accomplished. A normal distribution of HbF values for individual bursts has been reported (12). The mean was 65% HbF. Both of our umbilical cord samples produced bursts with skewed distributions of per cent HbF. The means were 30% and 69% in two different samples. This appeared to reflect the extent to which the HbF to HbA switch had progressed in the two neonates.

Most bursts of adult origin synthesized large amounts of HbA, appropriate for the developmental stage of the precursor cells. In contrast, to a normal distribution of values of per cent HbF reported for adult origin bursts (13) we found considerable skewing toward lower percentages of HbF. It is possible that the inability to measure hemoglobin in 12 of the 15 day bursts has contributed to this result. However, the skewing is even more apparent among the bursts studied at 18 days only one of which contained too little hemoglobin to measure. Ratios of \( \gamma/(\gamma + \beta) \) chain synthesis in similar cultures have yielded numbers between 0.055 and 0.57 in studies done between culture day 11 and 16 (8, 9, 11, 13, 15).

These studies provide evidence that bursts from fetal, neonatal and adult sources accumulate decreasing levels of HbF in parallel with the decrease in circulating HbF at those developmental stages. The standard deviation of the mean percentage of HbF for bursts from each source also reflects the heterogeneity of the hemoglobin phenotype at each developmental stage. The greatest diversity with respect to levels of HbF produced was among umbilical cord bursts which are present during the perinatal in vivo switch from primarily HbF to primarily HbA production. Bursts from a fetal liver source demonstrated the least diversity with respect to the percentages of HbF which bursts synthesized. Bursts of adult origin were intermediate with regard to this variable.

**Differences among Precursors from a Single Developmental Stage—**Mature bursts of adult origin harvested on culture day 15 contained an average of 28% HbF; Colorless bursts which were left in situ on day 15 and harvested when mature on day 18 contained an average of 13% HbF. The finding that the average percent of HbF present in adult bursts mature on day 15 decreased by almost a factor of 2 for bursts mature on day 18 suggests that there are differences in commitment to synthesis of HbF among sequentially maturing waves of erythroid bursts. Similar findings have been mentioned in previous studies of \( \gamma/(\gamma + \beta) \) ratios in cultures of fetal and neonatal precursors (14, 16, 37). Of the bursts studied at 15 days, 12 (24%) yielded unusable results since the levels of hemoglobin were too low to detect. It is possible that these bursts contain lower percentages of HbF than bursts in this group which contain measurable hemoglobin levels. Thus, the 25% HbF mean obtained for this group may be high.
However, even if the 12 bursts contained only HbA, which we consider to be unlikely, the mean for the entire group would still be higher than the 15% mean obtained for the group harvested on culture day 18. Thus, these studies suggest that populations of bursts maturing at later times in culture synthesize decreasing percentages of HbF.

The findings we have described are consistent with the hypothesis that hemoglobin switching is accomplished by the proliferation of successive waves of erythropoietic precursors or to biochemical changes within all differentiating stem cells.

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