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Hemoglobin switching in culture: Evidence for a humoral factor that induces switching in adult and neonatal but not fetal erythroid cells

(Hb F regulation/erythroid stem cells/interactions)

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ABSTRACT **An erythropoietic activity that exerts a profound effect on fetal Hb synthesis is present in fetal sheep sera and it attains a peak concentration at the end of the second to the middle of the third trimester of fetal life. The activity consistently inhibits the increased synthesis of fetal Hb in cultures of burst-forming units (BFUes) from normal adults. In cultures of BFUes from homozygous β^+-thalassemias the activity produces a striking decline in γ chain synthesis, a decline in $^{G}\gamma/^{A}\gamma$ chain synthesis ratio, and an increase in δ/γ and α/non-α ratios—i.e., findings suggesting a genuine γ-to-β switch. The activity accelerates Hb F-to-Hb A switching in neonatal BFUe cultures but it has no effect on fetal Hb synthesis in cultures of BFUe obtained from human fetuses. These findings provide direct evidence that (*a*) humoral factors play a role in the regulation of the switch from fetal to adult Hb formation, and (*b*) progenitor cells from various stages of ontogeny respond differently to these factors. The results are compatible with the hypothesis that Hb switching during development is mediated through a change in a developmental program which controls the responsiveness of progenitor cells to "switching" activities in their environment.**

The switches of Hb expression during ontogeny provide a model system for studying regulation of gene activity in development and differentiation. Normally, in the erythroid cells of human, primate, sheep, and bovine fetuses, predominantly Hb F ($\alpha_2\gamma_2$) is synthesized and there is a switch from fetal to adult (Hb A, $\alpha_2\beta_2$) Hb formation at the perinatal period. Insights into the mechanism underlying Hb F-to-Hb A switching have been obtained from *in vitro* studies using clonal erythroid cultures (1, 2); these studies have shown that switching is controlled at the level of erythroid progenitors and stem cells (1–5). It has been proposed that programs influencing globin gene expression are encoded in stem cells and progenitors and that, during ontogeny, intrinsic or interactive changes in these programs result in Hb switching (6).

The purpose of the studies reported here was to test whether interactions between environment and cells play a role in Hb switching. Our studies were done in cultures of burst-forming units (BFUes) derived at various stages of human development. BFUes represent a class of immature erythroid progenitors which, in semi-solid cultures, form clones usually comprised of several thousand erythroblasts (erythroid bursts). In cultures of BFUes from adult individuals, moderate levels of Hb F are produced; cultures of fetal or neonatal BFUes synthesize levels of Hb F within the range of values expected for the fetal or neonatal stage of Hb ontogeny (1, 5, 7). We tested whether potential humoral inducers of Hb switching appear in sheep fetuses before or at the time Hb switching starts. We found that sera obtained from the ontogenetic time preceding the Hb switching in sheep have a profound effect on Hb F synthesis in human adult BFUe cultures. They influence Hb F-to-Hb A switching in neonatal BFUe cultures but have no effect on fetal BFUes.

These results suggest that factors in the hemopoietic environment play a role in the switching process. Our findings further show that the response of hemopoietic cells to these environmental factors depends on the stage of ontogeny from which the cells derive. The simplest interpretation of these observations is that the cellular regulation of Hb switching is mediated through qualitative changes that occur in progenitor cells; as development progresses, the progenitor cells may acquire the ability to interact with activities present in their environment and this interaction either directly or indirectly (through changes in progenitor cell differentiative programs) brings about a switch of the program of globin synthesis in the erythroid cells.

METHODS

The cells used for culture were from peripheral blood or bone marrow of adult volunteers, from umbilical cord bloods of neonates, and from livers or fetal blood of abortuses. Peripheral blood mononuclear cells were isolated through Ficoll-sodium Metrizoate (Lymphoprep, Nyegaard, Norway) centrifugation (1). Bone marrow cells were obtained from buffy coat preparations. Fetal liver cells were prepared as a single-cell suspension from liver fragments after removal of cell aggregates and stromal pieces (5). Appropriate cell concentrations ($1–3 \times 10^5$/ml) were cultured in methylcellulose plates in the presence of erythropoietin (2 international units/ml), 10 μM 2-mercaptoethanol, 0.1% bovine serum albumin, and 30% (vol/vol) serum. To study the effect of fetal sheep serum, sets of duplicate cultures, one grown in the presence of fetal calf serum and the other in the presence of fetal sheep serum, were used. Growth was monitored by inspecting the plates at desired intervals for colony maturation.

For measuring globin biosynthesis, fully hemoglobinized erythroid clones were lifted from the plates with ultrathin pipettes, pooled, and incubated with [^{3}H]leucine as described (8). Lysates from cultured erythroblasts were subjected to isoelectric focusing and the relative synthesis of globin chains was estimated from densitometric tracings of the resultant fluorograms (8). In several experiments, fluorescent antibodies were used to evaluate the expression of Hb F and Hb A in single

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Abbreviations: BFUe, burst-forming unit; HPFH, hereditary persistence of fetal Hb.

bursts. Approaches described in detail elsewhere (1) were used with the exception that, in addition to monospecific anti-Hb F and anti-A antibodies, anti-γ or anti-β chain monoclonal antibodies were used for fluorescent labeling.

RESULTS

Sera or plasmas from sheep fetuses contain an activity that supports the growth and maturation of human BFUes in culture. By using sera from fetuses whose gestational age was known, we found that the optimal concentration of this activity is reached between 80 and 120 days of gestation. Because Hb F-to-Hb A switching in sheep usually starts after the 120th day of gestation (9), the 80- to 120-gestational day-old serum will be referred to as "preswitch" serum. When preswitch serum was used in culture, cloning efficiency of bone marrow BFUes was 2.34 times higher (n = 27; SEM = 0.29) compared to controls, and that of peripheral blood BFUes was 2.14 times higher (n = 31; SEM = 0.21) than in controls. In these cultures, size of erythroid clones (average number of cells per erythroid burst) was 4.2 times higher (n = 20; SEM = 0.5) than in controls.

The effect of preswitch serum on Hb F synthesis was first noted in cultures of adult BFUes. In cultures in the presence of fetal calf serum there was a consistent stimulation of Hb F synthesis. When preswitch serum was used instead of the standard medium in culture, Hb F synthesis was strikingly decreased (Fig. 1). The inhibition of Hb F synthesis was observed when either bone marrow or peripheral blood BFUes from normal persons were used. α/non-α synthesis ratios remained balanced and there was a slight decline in $^{G}\gamma/^{A}\gamma$ ratio. Hb F synthesis was inhibited in cultures of BFUe from individuals with sickle cell anemia (Fig. 1 *Right*) or other conditions in which there is an acquired increase of fetal Hb. The effects of preswitch serum on Hb F synthesis were apparent when fluorescent antibodies were used to detect Hb F in individual clones. Whereas in cultures of normal BFUe the majority of erythroid bursts display a bimorphic Hb F expression with several subclones labeled by anti-Hb F fluorescent antibodies (1, 8), bursts grown under the influence of preswitch serum either failed to show anti-Hb F-labeled cells or they contained only a few scattered Hb F erythroblasts.

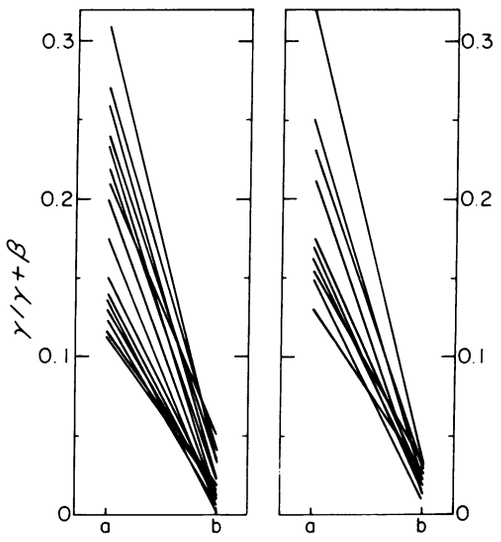

FIG. 1. Effect of preswitch serum on Hb F synthesis in adult BFUe cultures: measurements of γ-globin synthesis (expressed as $\gamma/\gamma + \beta$ ratio) in cultures done with standard medium (point a) or with preswitch sheep serum (point b). Results from experiments in which $\gamma/\gamma + \beta$ ratios in the control cultures were >0.1 are plotted. (*Left*) Cultures of BFUe from nonhemoglobinopathic persons with normal Hb F *in vivo*. (*Right*) Cultures of homozygous Hb S BFUes.

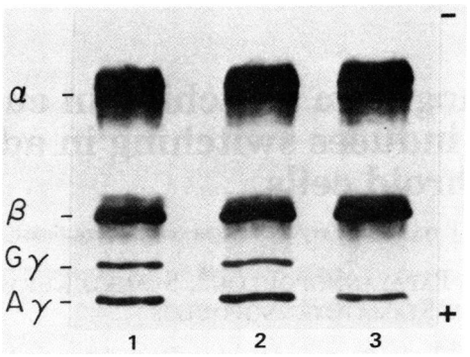

FIG. 2. Effect of preswitch serum on $^{G}\gamma$ chain synthesis in Greek ($^{A}\gamma$) HPFH BFUe cultures. Lanes: 1 and 2, globin isoelectric focusing of cell lysates from cultures with standard medium; 3, globin isoelectric focusing in cultures with preswitch serum. Note the absence of $^{G}\gamma$ chain synthesis in lane 3.

To test further the effect of preswitch serum on Hb F synthesis, BFUes from a heterozygote with the $^{A}\gamma$ (Greek) variant of hereditary persistence of fetal Hb (HPFH) were used for culture. In Greek $^{A}\gamma$ HPFH, 12–20% of the Hb synthesized *in vivo* is fetal and 92–96% of the γ chains are $^{A}\gamma$ (10). In BFUe cultures, synthesis of Hb F increases 2- to 3-fold over the *in vivo* level and, most importantly, 30–35% of the γ chains are $^{G}\gamma$ (10). When $^{A}\gamma$ HPFH BFUes were cultured in the presence of preswitch sheep serum, the level of Hb F synthesis in culture was similar to the level *in vivo* and $^{G}\gamma$ chains were not detectable (Fig. 2). This finding provided qualitative evidence that the preswitch sheep serum inhibits the partial β-to-γ switch that takes place in the cultures of adult BFUes.

Enhancement of γ-chain synthesis and a decrease in α/non-α chain ratio (compared to the *in vivo* levels) have been observed in cultures of homozygous β-thalassemia BFUes (11). We cultured homozygous β^{+}-thalassemic BFUes under standard conditions or in the presence of preswitch serum. The preswitch serum produced a striking decline in γ chain synthesis (Fig. 3). In addition, the $^{G}\gamma/^{A}\gamma$ chain synthetic ratio changed in favor of $^{A}\gamma$ chains and there was a striking increase in δ/γ ratio, expected if a shift in expression from the γ to δ–β genomic region had occurred. Furthermore, the preswitch serum increased the α/non-α ratios, an expected finding because in these β^{+}-thalassemia cells a switch from γ to β chain synthesis will not produce a significant increment in synthesis

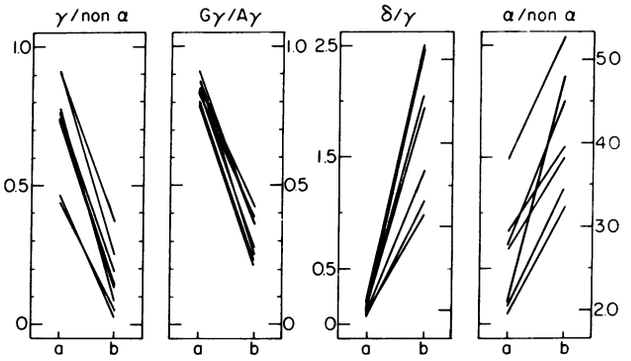

FIG. 3. Effects of preswitch serum on globin synthesis in cultures of homozygous β^{+}-thalassemia BFUes. Note the decline in γ/non-α and $^{G}\gamma/^{A}\gamma$ ratios and the parallel increment in δ/γ and α/non-α ratios. Points a and b as in Fig. 1.

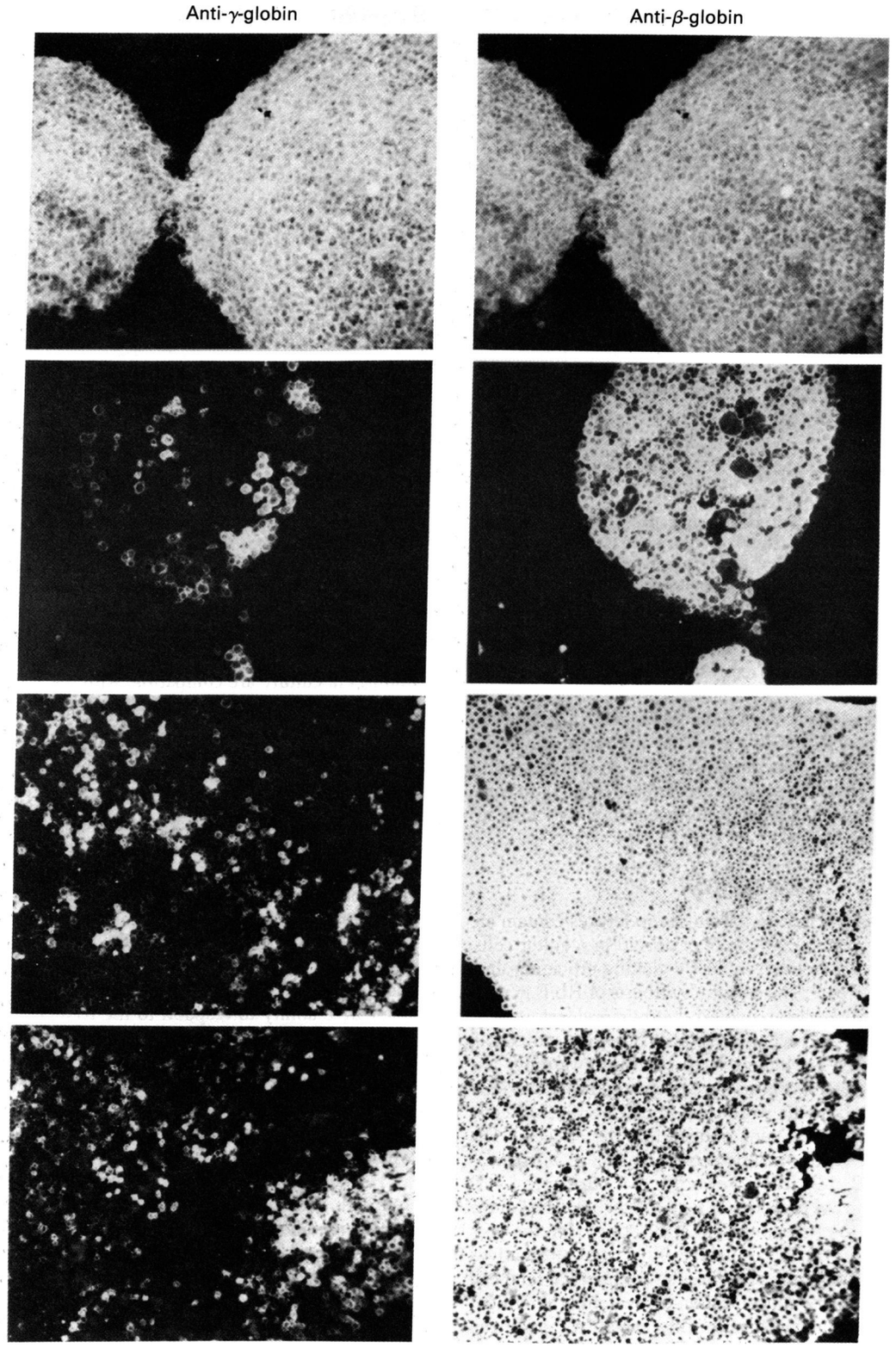

FIG. 4. Double-immunofluorescence labeling of erythroid bursts with anti-γ-globin monoclonal antibody conjugated to fluorescein isothiocyanate and anti-β-globin monoclonal antibody conjugated to rhodamine. The bursts are from cultures of homozygous β^+ thalassemia BFUes. Top pair represent a burst obtained from a culture done with standard medium. Note the uniform expression of fetal Hb. This uniform cellular expression of Hb F was typical of all the bursts examined by immunofluorescence. The bottom three pairs represent bursts obtained from a culture with preswitch serum. Note that only scattered cells or sectors of a burst are labeled for Hb F. This finding was typical of the bursts present in the cultures with preswitch serum.

of β chains. The appearance of erythroid bursts labeled with the anti-γ antibody was characteristic. Although in the control cultures the cells of the bursts were labeled more or less uniformly, in cultures with preswitch serum only small areas of bursts were

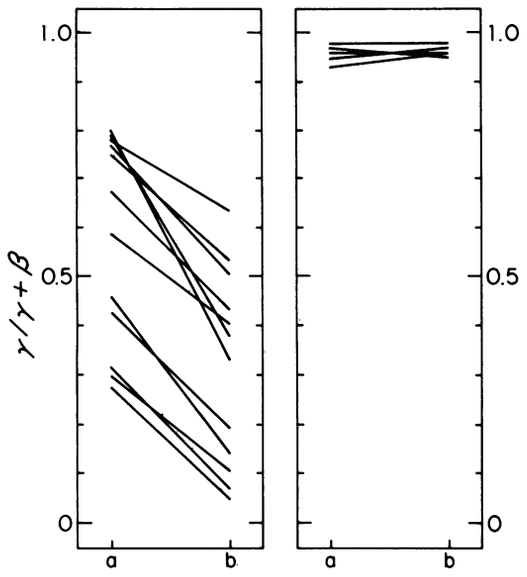

FIG. 5. Effects of preswitch serum on neonatal BFUe cultures (*Left*) and fetal BFUe cultures (*Right*). Points a and b are as in Fig. 1. Note the consistent decline in γ chain synthesis in the neonatal cultures and the absence of effect of preswitch serum in fetal BFUe cultures.

labeled with the antibody (Fig. 4). These data suggest that a genuine switch in non-α globin expression took place in the thalassemia cells cultured in preswitch fetal serum.

When standard media are used, the levels of Hb F synthesis in cultures of neonatal BFUes are within the range of values observed in the *in vivo* cells of neonates (8). In cultures with preswitch serum, Hb switching was accelerated (Fig. 5). In contrast, the preswitch serum failed to induce γ-to-β switching in the cultures of fetal BFUes.

DISCUSSION

These findings suggest that sera from sheep fetuses contain an activity that amplifies the growth of erythroid progenitor cells (as indicated by an increment in BFUe cloning efficiency and clone size) and decreases the relative synthesis of Hb F in erythroid clones. These findings suggest that humoral factors play a role in Hb switching. Previous attempts to identify factors that induce Hb F-to-Hb A switching have been unrewarding. No changes in globin chain synthesis have been noted when various types of conditioned media were used in culture (6), and contradictory information has been obtained on the effects of Hb F synthesis of the erythropoietic activity known as burst-promoting activity (12, 13). It is noteworthy that our control cultures were grown in the presence of fetal calf serum. The failure of fetal calf serum to produce the effect observed with the preswitch sheep serum can be explained by several alternatives such as the gestational time at which the commercial fetal calf serum was obtained, the concentration of "switching activities" present in such serum, or species differences. Although the physiological significance of the activity we describe, its biochemical nature, the cellular level of its action, and the mechanism of its action remain to be determined, the observations summarized here are of direct relevance to the question of Hb switching because they show that interactions between erythroid cells and their environment are involved in the control of this phenomenon.

Previous work on Hb switching in culture has focused on the role of progenitor cells in this process. Studies in clonal cultures of erythroid progenitors from various stages of ontogeny suggest

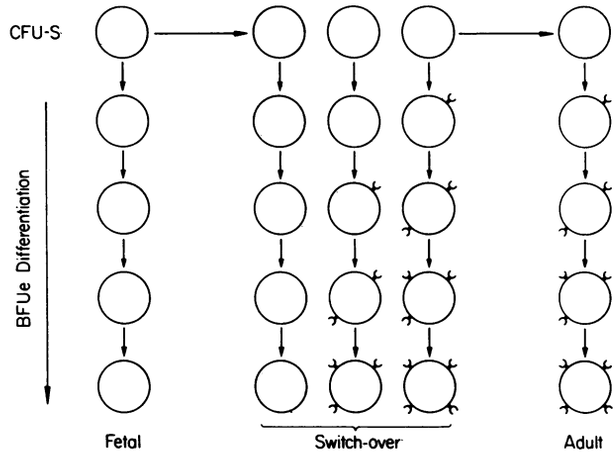

FIG. 6. Diagram of the hypothesis that environment–cell interactions are involved in the switch from Hb F to Hb A formation during ontogeny. It is postulated that a stem cell (CFU-S) program directs the formation of receptors which interact with switching activities in the hemopoietic environment. Activation of this program during the course of development makes the committed cells responsive to switching factors, like the one we describe here.

that, in these cells, programs of globin expression that are specific for each stage of ontogeny are encoded (1–7, 14, 15). "Commitment" of progenitor cells to such programs appears to be determined early in hemopoietic cell differentiation (6). The findings in culture are compatible with the hypothesis that Hb switching is controlled through changes in developmental programs that take place in multipotent or pluripotent stem cells (6). In our experiments we observed that the preswitch serum had no effect in the cultures of fetal progenitors, induced switching in cultures of neonatal progenitors, and inhibited γ chain synthesis in normal adult BFUe cultures. These observations are compatible with the possibility that switching is controlled through a change in a stem cell program which directs the appearance, in the committed progeny, of a property (a cellular or nuclear receptor?) influencing the response of a cell to an inductive environment (Fig. 6). Fetal cells cannot switch because they cannot respond to switching factors present in their environment; neonatal cells switch in proportion to their qualitative ability to respond to the factors present in the environment. If the property of the progenitor cells that controls the interaction between the cells and their environment appears late in the differentiation of BFUe, the observed inverse relationship between degree of differentiation of adult progenitors and their potential to form Hb F-expressing cells (1) would be expected.

The detection of a humoral activity that influences the switch from Hb F to Hb A formation has apparent consequences on experimentation on the biology of Hb switching. Thus far, studies of the cellular control of Hb switching were limited on assessing the commitments or "potentials" of progenitor cells. Availability of humoral activities that induce Hb switching and their eventual purification will allow direct studies of the mechanisms that control Hb switching at the cellular and molecular levels.

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