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Globin chain synthesis in single erythroid bursts from cord blood: Studies on $\gamma \rightarrow \beta$ and $^{G}\gamma \rightarrow {}^{A}\gamma$ switches

(Hb switch/$^{G}\gamma$-to-$^{A}\gamma$ and γ-to-β ratios/erythroid burst-forming unit/linked genes)

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ABSTRACT **Erythroid bursts from cord or adult blood were grown in methylcellulose cultures (3 international units of erythropoietin per plate). On day 13, single bursts were picked up and reincubated for 16–24 hr with [^{3}H]leucine. Radioactive globin chains [α, β, $^{G}\gamma$, and $^{A}\gamma$ (Ala-136)] were analyzed by either isoelectric focusing on polyacrylamide gels and fluorography or carboxymethylcellulose chromatography. In all cases, α to non-α globin radioactivity ratios were close to 1. In single cord blood bursts, the values of both γ-to-β and $^{G}\gamma$-to-$^{A}\gamma$ ratios were spread over a large spectrum and further characterized by a continuous rather than a bimodal distribution. Moreover, the $^{G}\gamma$-to-$^{A}\gamma$ ratios demonstrated in single bursts appeared to be directly correlated with the respective γ-to-β ratios. These data suggest that both the $\gamma \rightarrow \beta$ and the $^{G}\gamma \rightarrow {}^{A}\gamma$ switches are mediated via mechanisms modulating the relative activities of the different genes in the non-α globin gene cluster rather than via selection of clones committed to the preferential synthesis of β and $^{A}\gamma$ globins. In contrast with the results obtained with cord blood, individual adult blood bursts synthesize a lower and hence relatively more uniform amount of γ globin chains.**

Previous studies have identified two types of human γ globin chains, which are respectively characterized by the presence of either glycine ($^{G}\gamma$) or alanine ($^{A}\gamma$) in position 136 (1). The synthesis of these chains is governed by two nonallelic structural genes (2, 3). Because glycine and alanine are neutral amino acids, the relative $^{G}\gamma$-to-$^{A}\gamma$ ratio has been evaluated by analysis of the glycine/alanine content of the cyanogen bromide fragment (CB3), which includes residue 136. It has been thereby established that the $^{G}\gamma$-to-$^{A}\gamma$ ratio, although fairly constant in the fetal period (approximately 7:3), declines progressively during the postnatal $\gamma \rightarrow \beta$ switch, leading to an average value of 2:3 in the small residual amount of Hb F detectable in normal adult blood (1–6). Therefore, two different types of switch operate in the postnatal period—the $\gamma \rightarrow \beta$ and the $^{G}\gamma \rightarrow {}^{A}\gamma$ switches. Although the former one has been extensively investigated (7–10), studies on the latter one have been hampered so far by lack of a simple analytical method to separate $^{G}\gamma$ from $^{A}\gamma$ chains. Therefore, it is not yet established whether the $\gamma \rightarrow \beta$ and the $^{G}\gamma \rightarrow {}^{A}\gamma$ switches operate via either selection of different cellular clones or modulation of phenotypic expression of different genes. Elucidation of this mechanism, however, is relevant from both a theoretical and a clinical point of view. Indeed, previous reports indicate that unusually high Hb F levels in adult patients with various types of hemoglobinopathies may be associated with amelioration of their clinical condition (11, 12).

In an attempt to elucidate the mechanism(s) underlying the $\gamma \rightarrow \beta$ and the $^{G}\gamma \rightarrow {}^{A}\gamma$ switches, two recently developed techniques have been employed here: (*i*) evaluation of globin chain synthesis in individual colonies (13) (see *Methods*), each derived from a single circulating erythroid precursor (erythroid burst-forming unit, BFU-E) (14–16) and (*ii*) isoelectric focusing (IEF) (17–19) in the presence of the detergent Nonidet P-40. This technique separates $^{G}\gamma$ from $^{A}\gamma$ globin chains, thus allowing an evaluation of their relative synthetic rates (18, 19).

By these methods it is shown here that single bursts from cord blood differ widely in their relative ability to synthesize $^{G}\gamma$, $^{A}\gamma$, and β globin chains. Furthermore, a direct correlation is demonstrated between γ-to-β and $^{G}\gamma$-to-$^{A}\gamma$ synthetic ratios in individual cord blood bursts.

METHODS

Human Subjects. Heparinized blood was obtained from the cord of normal newborns and the anticubital vein of normal adult volunteers.

Erythropoietin (Ep) Purification. The urine from a patient with pure erythrocyte aplasia was concentrated against polyethylene glycol at 4°C and lyophilized (specific activity, 20–27 international units (IU)/mg of protein). This crude Ep preparation was further purified by means of a three-step chromatography method (20) (final specific activity, ≈500 IU/mg of protein). The partially purified Ep, virtually free of both activity-stimulating myeloid-macrophage colonies and inhibitor(s) of erythroid colony formation, was employed as the stimulus for BFU-E. It must be further emphasized that endotoxin was virtually absent in this Ep preparation [i.e., the *Limulus* assay (21) showed the presence of as little as 0.01 ng of endotoxin per 1 IU of Ep].

Erythroid Burst Culture. The mononuclear cells were harvested by using a slight modification (22) of the original Ficoll–Isopaque technique described by Böyum (23) and cultured according to the method reported by Iscove (20), as modified by Peschle *et al.* (24). Thus, each 1-ml plate contained the following components in Dulbecco's modified Eagle's medium¶: methylcellulose (0.8% final concentration), α-thioglycerol (0.1 mM), fetal calf serum (40%), 1–3 × 10^{5} nucleated cells, and Ep (3 IU). The plates were incubated in a humidified 7.5% CO_2/92.5% air atmosphere at 37°C. BFU-E colonies containing a

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Abbreviations: BFU-E, erythroid burst-forming unit; IEF, isoelectric focusing; Ep, erythropoietin; CMC, carboxymethylcellulose; Hb, hemoglobin; IU, international units.

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¶ Dulbecco's medium was modified here to contain L-alanine (25 μg/ml), L-asparagine·H_2O (50 μg/ml), L-aspartic acid (30 μg/ml), L-cysteine (70 μg/ml), L-glutamic acid (75 μg/ml), L-proline (40 μg/ml), sodium pyruvate (110 μg/ml), vitamin B_{12} (0.025 μg/ml), biotin (0.03 μg/ml), and Hepes buffer (2.5%) (1 M, GIBCO).

minimum of 400–600 cells were scored or incubated with radioactive amino acids on day 13. The identification was performed *in situ* on the basis of their orange-red color.

Radioactive Labeling of Globin Chains. In experiments involving labeling of pooled bursts, 25–50 μCi of [^{3}H]leucine (Amersham, 55–137 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) was added to each culture dish on day 13. After a 16- to 24-hr incubation period, all well-hemoglobinized BFU-E colonies were picked up and washed three times (first in 10 ml of physiologic saline, containing 2.5 × 10^6 carrier cord blood erythrocytes, then in 10 and finally in 1.2 ml of saline) at 4°C. The resulting pellet was kept frozen until analyzed.

In experiments involving labeling of individual well-hemoglobinized colonies, single bursts, visualized through an inverted microscope, were randomly picked up by means of a fine Pasteur pipette in a 2- to 3-μl volume, transferred into 10 μl of culture medium containing 25–150 μCi of [^{3}H]leucine, further incubated for 16–24 hr, washed as above in 1.2 ml of saline (containing 2.5 × 10^6 erythrocytes), and stored at −30°C.

Globin Preparation and Analysis. Globin, prepared from stroma-free lysates by the acid acetone procedure (25), was analyzed by means of either carboxymethylcellulose (CMC) chromatography (25) or IEF in the presence of Nonidet P-40 (17, 18). Gel fluorography (26, 27) and scanning of the exposed film were performed as described (17, 18).

RESULTS

Erythroid bursts from Ficoll-separated blood cells were grown in viscous methylcellulose cultures. This method allowed us to pick up a single BFU-E colony, which was then incubated in the presence of high concentrations of radioactive amino acid, thus yielding an adequate amount of labeled Hb for the quantitative analysis of globin chains. Preliminary experiments were performed to establish the appropriate culture conditions. On day 13, the number of bursts from either adult or cord blood reached virtually plateau levels in the presence of 0.4–3.0 IU of Ep per plate (Table 1). At this time, however, the rate of Hb synthesis appeared to be optimal in the presence of 2–3 IU of Ep (experiments not shown). On the basis of these observations, bursts were grown in all experiments in the presence of 3 IU of Ep per plate through a 13-day culture period, which was followed by the 16- to 24-hr incubation with radioactive amino acid. Table 2 and Fig. 1 *Lower* show the results obtained by CMC chromatography of labeled globin chains synthesized by cord blood bursts. Either single colonies or the pool of all bursts grown in a single dish were analyzed. In both cases, the α-to-non-α globin ratio is close to 1, as expected for normal globin synthesis, thus indicating that no extraneous material contaminates the globin preparation to a significant extent. This observation is further supported by both fingerprint analysis of purified globin chains and lack of any change in the α-to-non-α ratio after either Sephadex G-200 chromatography in formic acid (28) or DEAE 52 (29) purification of Hb (results not presented here). Furthermore, the β-to-β globin ratio in the pooled colonies was approximately 2:1 (Table 2), a value similar

Table 1. Number of BFU-E colonies from six adult and four cord blood samples after incubation with graded amounts of Ep

Ep, IU/plate	Adult blood, BFU-E/ 3 × 10^5 cells	Cord blood, BFU-E/ 10^5 cells
0.00	1 ± 0.6	1 ± 1.3
0.01	2 ± 0.7	3 ± 1.2
0.04	20 ± 4.9	17 ± 2.5
0.10	49 ± 8.0	32 ± 5.3
0.40	74 ± 3.8	66 ± 6.7
1.00	71 ± 3.3	72 ± 6.9
3.00	69 ± 5.7	73 ± 5.0

Mean ± SEM of mean values from six adult and four cord blood samples, each evaluated in duplicate plates.

Table 2. Globin chain synthesis in cord blood bursts

Globin source	Method	Radioactivity ratios α/non-α	β/α	γ/α
Pooled colonies	CMC	1.00	0.31	0.69
	IEF	1.05	0.29	0.65
Colony 1	CMC	1.17	0.48	0.37
	IEF	1.09	0.46	0.39
2	CMC	1.12	0.40	0.49
	IEF	0.99	0.39	0.57
3	CMC	0.93	0.83	0.25
	IEF	1.03	0.78	0.19
4	CMC	1.08	0.50	0.42
	IEF	1.10	0.45	0.45
5	CMC	1.17	0.38	0.47
	IEF	1.10	0.31	0.59
6	CMC	1.09	0.44	0.47
	IEF	0.95	0.43	0.65
7	CMC	1.22	0.43	0.39
	IEF	1.07	0.39	0.55
8	CMC	1.09	0.20	0.71
	IEF	0.98	0.18	0.83
9	CMC	0.97	0.20	0.83
	IEF	1.08	0.15	0.79
10	CMC	1.11	0.32	0.58
	IEF	1.09	0.27	0.65

Globins from colonies 1–10 are shown in Fig. 2, lanes 2–11; globins from pooled colonies are shown in Fig. 2, lane 1.

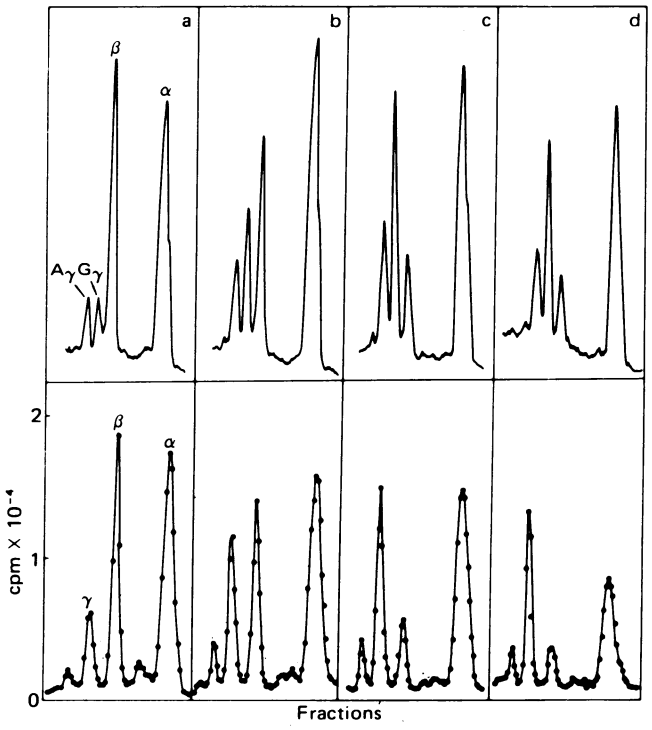

FIG. 1. Quantitation of radioactive globins synthesized by single cord blood bursts. (*Upper*) Densitometric scans of the IEF pattern shown in the fluorogram of Fig. 2; *a*, *b*, *c*, and *d* correspond to lanes 4, 5, 9, and 10 respectively. (*Lower*) CMC chromatography of radioactive globin from the same colonies as above.

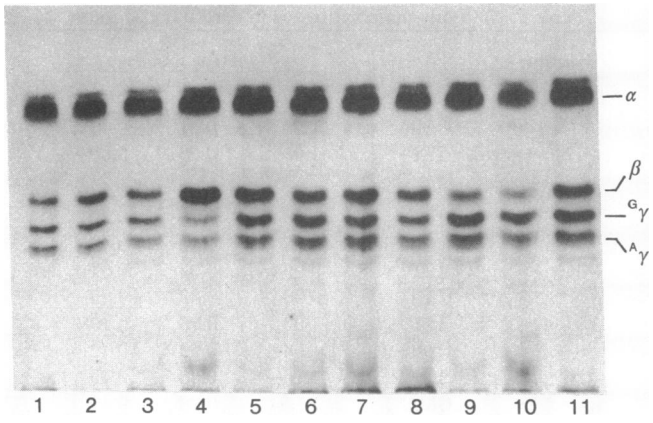

FIG. 2. Fluorogram of the IEF pattern of globin synthesized in pooled colonies (lane 1) and cord blood single bursts (lanes 2–11).

to that obtained after incubation of cord blood. However, the relative synthesis of globin chains in individual bursts showed a striking variation, in that the γ-to-β ratio in single colonies ranged from approximately 0.2 up to 5.0 (Table 2).

IEF in the presence of Nonidet P-40 allows a clear-cut separation of α, β, $^{G}\gamma$, and $^{A}\gamma$ globin chains (17–19). The fluorographic pattern induced by radioactive globin from cord blood bursts is shown in Fig. 2 and some scans are shown in Fig. 1 *Upper*. In each case four major radioactive bands appear at pIs of 7.8, 7.2, 6.95, 6.85, the values expected for α, β, $^{G}\gamma$, and $^{A}\gamma$ chains, respectively. Once again, the α-to-non-α ratio was approximately 1:1 in all tested colonies, thus confirming the CMC data. In addition, the $^{G}\gamma + {}^{A}\gamma$-to-β proportion was essentially identical to the CMC-determined γ-to-β ratio; both separation techniques yielded similar values for both individual and pooled bursts (Table 2 and Fig. 1).

The possibility that the high Hb F levels and their wide spread in cord blood colonies constitute an experimental artifact, due to the particular culture conditions, is rendered unlikely by the results obtained for individual bursts from adult blood. The adult colonies synthesized a lower (2–14% in six normal adults) and hence relatively more uniform percentage of γ chains (Fig. 3 and unpublished observations).

It seemed of interest to correlate $^{G}\gamma$-to-$^{A}\gamma$ and γ-to-β synthetic ratios in individual cord blood colonies. As seen in Fig. 4, a significant direct correlation exists between these two parameters (Fig. 4 *Left* and *Right*, $P < 0.01$; Fig. 4 *Center*, $P < 0.05$), thus indicating that colonies synthesizing preferentially

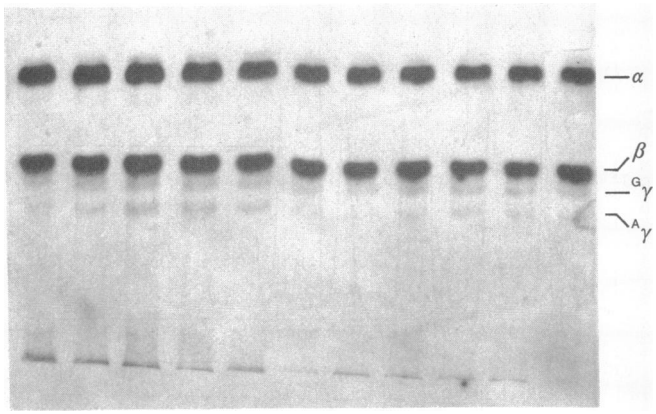

FIG. 3. Fluorogram of the IEF pattern of globin synthesized in single bursts from adult blood.

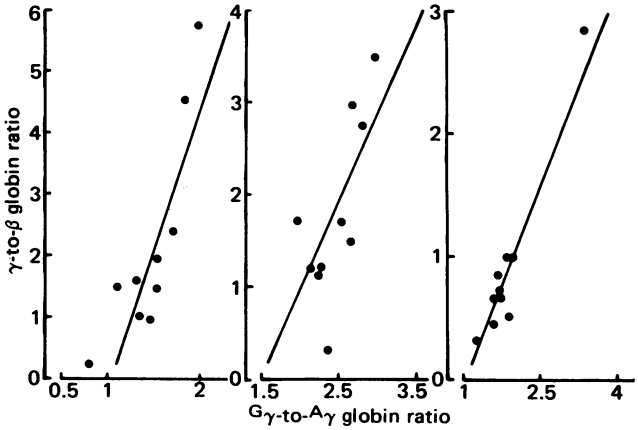

FIG. 4. γ/β versus $^{G}\gamma/^{A}\gamma$ in single bursts from cord blood from three different newborns. The regression of γ/β on $^{G}\gamma/^{A}\gamma$ is significant. (*Left*) r = 0.83; $P < 0.01$. (*Center*) r = 0.71; $P < 0.05$. (*Right*) r = 0.97; $P < 0.01$).

γ globin show a higher γ-to-$^{A}\gamma$ ratio than those producing mainly β chains. This relationship has been further observed in additional experiments on individual bursts from peripheral blood of a 2-week-old neonate (results not presented here). A low $^{G}\gamma$-to-$^{A}\gamma$ ratio is also apparent in the small amount of γ chains synthesized by adult bursts (Fig. 3 and unpublished results).

DISCUSSION

Blood films from normal adult individuals, treated with fluorescent anti-Hb F antiserum, reveal the presence of Hb F in only 0.2–7.0% of erythrocytes (30, 31): the Hb F content of these erythrocytes, however, is sufficiently elevated to account for the total amount of Hb F in adult blood (<1%) (30). This well-established observation indicates that the γ → β switch operates via complete suppression of γ chain synthesis in a large majority of the erythrocyte precursors and partial blockade of its production in the residual ones. Although the mechanism(s) underlying the γ → β switch has not been elucidated, two basic hypotheses can be considered. The first one envisions the existence of erythroid clones irreversibly programmed to produce exclusively (or predominantly) either Hb F or Hb A—i.e., the switch is mediated via selection of clones already programmed to produce either solely or largely Hb A. The second hypothesis postulates a modulation of Hb synthesis within a single pool of erythrocyte precursors via mechanism(s) operating at the molecular level to repress γ and derepress β chain syntheses.

The present studies with cord blood BFU-Es indicate that the values of the γ-to-β ratio in individual bursts (known to derive from single BFU-Es) (9, 14, 15) are spread over a large spectrum and are further characterized by a continuous rather than a bimodal distribution.‖ These data, which are consistent with older, semiquantitative observations that cord blood erythrocytes contain both Hb A and Hb F during the switch (33–35), are hardly compatible with the clonal selection hypothesis; rather they support the concept that a not-yet-identified perinatal mechanism(s) modulates progressively the erythrocyte precursor to synthesize predominantly or exclusively Hb A. The present results, however, do not distinguish whether the modulation mechanism operates alone or in association with a selection mechanism.

‖ After completion of this manuscript Kidoguchi *et al.* (32) reported, by means of a different technique, a similar spread of Hb F synthetic values in cord blood bursts.

Although the $\gamma \rightarrow \beta$ switch has been extensively investigated, far less information is available on the $^{G}\gamma \rightarrow {}^{A}\gamma$ switch, due to inability of present analytical methods to separate $^{G}\gamma$ and $^{A}\gamma$ chains. The latter switch may be mediated by a number of mechanisms: (*i*) $^{G}\gamma$ and $^{A}\gamma$ chains could be produced in different cell lineages—i.e., variation of their ratios simply derives from the predominance of either of these clones. (*ii*) Alternatively, the $^{G}\gamma$ and $^{A}\gamma$ chains could be produced uniformly in all erythrocyte precursors; the switch would operate at either the transcriptional or translational level by modifying the relative synthesis of the two chains. (*iii*) $^{G}\gamma$ and $^{A}\gamma$ chains could be produced in all cell clones in heterogeneous proportions; accordingly, mechanisms acting via molecular modulation and cellular selection would operate cooperatively. Once again, our data suggest that a modulation mechanism underlies the $^{G}\gamma \rightarrow {}^{A}\gamma$ switch, because both $^{G}\gamma$ and $^{A}\gamma$ are present in different proportions in various colonies.

Finally, it is emphasized that the direct correlation between $^{G}\gamma$-to-$^{A}\gamma$ and γ-to-β ratios suggests a linkage between the two switches. The molecular mechanisms underlying this phenomenon are unknown, but it is tempting to speculate that a single regulatory mechanism might simultaneously modulate the whole nongene cluster ($^{G}\gamma/^{A}\gamma/\gamma/\beta$) (36) by acting directly either on chromatin conformation (37–39) or transcription of the linked genes, thus leading to the observed correlation between $^{G}\gamma$-to-$^{A}\gamma$ and γ-to-β ratios. Alternatively, two different mechanisms might operate simultaneously on the $^{G}\gamma/^{A}\gamma$ and γ/β clusters, respectively. In this regard, the slope difference observed for various cord blood samples may reflect asynchrony of the two switches in different individuals. This problem and the impact of hereditary factors (40) in affecting the $^{G}\gamma$-to-$^{A}\gamma$ ratio should be assessed in future work.

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