

Cellular Mechanisms for Increased Fetal Hemoglobin Production in Culture: *EVIDENCE FOR CONTINUOUS COMMITMENT TO FETAL HEMOGLOBIN PRODUCTION DURING BURST FORMATION*

George J. Dover, Makio Ogawa

J Clin Invest. 1980;66(5):1175-1178. <https://doi.org/10.1172/JCI109949>.

Rapid Publication

Using microscopic immunodiffusion assays and microdensitometric analysis of pericellular immunoprecipitate, the percentage of nucleated erythrocytes containing fetal hemoglobin (FNRBC) and the mean picograms of fetal or adult hemoglobin per nucleated erythrocyte (picograms HbF/NRBC, picograms HbA/NRBC) were assayed in 14-d-old colonies (bursts) derived from peripheral blood erythroid progenitors. In the peripheral blood of 11 normal adults only $2.2 \pm 0.5\%$ (mean \pm SE) erythrocytes contained HbF whereas pooled bursts from the same subjects revealed a 13-fold increase in the percentage of FNRBC ($29.6 \pm 3.9\%$). In culture both the picograms HbF/NRBC (5.2 ± 0.4) and the picograms HbA/NRBC (27.7 ± 1.5) are increased $\sim 20\%$ above the mean in vivo levels in NRBC from normal bone marrow aspirates. Analysis of each of 58 bursts from one subject demonstrated that FNRBC are present in all bursts and range from 5.0 to 95.0% of the total NRBC per burst. The percent FNRBC in each burst was neither correlated with picograms HbF/NRBC per burst nor with picograms HbA/NRBC per burst. Individual subcolonies from one burst in each of two subjects demonstrated between 3 and 81% FNRBC.

These findings indicate that first, the increase in HbF production in culture is primarily due to increased production of the number of cells containing HbF, not to increased picograms HbF/NRBC. Second, all 14-d bursts contain some FNRBC. Third, just as seen in vivo, the picograms HbF/cell and the number [...]

Find the latest version:

<https://jci.me/109949/pdf>



Cellular Mechanisms for Increased Fetal Hemoglobin Production in Culture

EVIDENCE FOR CONTINUOUS COMMITMENT TO FETAL HEMOGLOBIN PRODUCTION DURING BURST FORMATION

GEORGE J. DOVER and MAKIO OGAWA, *Howard Hughes Medical Institute, Laboratory for Human Biochemical Genetics, Departments of Pediatrics and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; Veterans Administration Medical Center and Department of Medicine, University of South Carolina, Charleston, South Carolina 29403*

ABSTRACT Using microscopic immunodiffusion assays and microdensitometric analysis of pericellular immunoprecipitate, the percentage of nucleated erythrocytes containing fetal hemoglobin (FNRBC) and the mean picograms of fetal or adult hemoglobin per nucleated erythrocyte (picograms HbF/NRBC, picograms HbA/NRBC) were assayed in 14-d-old colonies (bursts) derived from peripheral blood erythroid progenitors. In the peripheral blood of 11 normal adults only $2.2 \pm 0.5\%$ (mean \pm SE) erythrocytes contained HbF whereas pooled bursts from the same subjects revealed a 13-fold increase in the percentage of FNRBC ($29.6 \pm 3.9\%$). In culture both the picograms HbF/NRBC (5.2 ± 0.4) and the picograms HbA/NRBC (27.7 ± 1.5) are increased $\sim 20\%$ above the mean *in vivo* levels in NRBC from normal bone marrow aspirates. Analysis of each of 58 bursts from one subject demonstrated that FNRBC are present in all bursts and range from 5.0 to 95.0% of the total NRBC per burst. The percent FNRBC in each burst was neither correlated with picograms HbF/NRBC per burst nor with picograms HbA/NRBC per burst. Individual subcolonies from one burst in each of two subjects demonstrated between 3 and 81% FNRBC.

These findings indicate that first, the increase in HbF production in culture is primarily due to increased production of the number of cells containing HbF, not to increased picograms HbF/NRBC. Second, all

14-d bursts contain some FNRBC. Third, just as seen *in vivo*, the picograms HbF/cell and the number of cells that contain HbF are independently regulated in culture. Fourth, commitment to produce HbF *in vitro* continues after subcolony formation in 14-d-old bursts. Augmentation of HbF production in culture therefore closely resembles that seen in acute erythroid stimulation *in vivo*.

INTRODUCTION

In normal adults peripheral blood erythropoietic progenitors, termed "burst-forming units" (BFU-E),¹ produce in culture more fetal hemoglobin (HbF) than erythroid precursors that develop *in vivo* (1). HbF production *in vivo* is increased by three independently regulated mechanisms; increased production of cells containing HbF (F cells), increased production of HbF/F cells, or preferential survival of F cells in comparison with erythrocytes that contain no HbF (2, 3). It is unknown to what extent these three different mechanisms contribute to increased HbF production in culture. Furthermore, it is unclear whether all peripheral blood BFU-E have the capacity to make HbF during differentiation. For example, while isoelectric focusing of hemoglobin indicates that HbF is present in all individual BFU-E colonies (bursts) (4), immunofluorescent staining of intact bursts with anti-HbF antibody (5) and radioimmunoassays of hemo-

An abstract of this work was submitted to the 22nd Annual Meeting of the American Society of Hematology, December 1979, in Phoenix, Ariz.

Dr. Ogawa is a Medical Investigator for the Veteran's Administration. Dr. Dover is the recipient of Research Career Development Award K04 AM 00689-01.

Received for publication 25 August 1980.

¹ *Abbreviations used in this paper:* BFU-E, burst-forming unit, erythroid; F cell, erythrocyte containing fetal hemoglobin; FNRBC, nucleated erythrocyte containing fetal hemoglobin; HbA, adult hemoglobin; HbF, fetal hemoglobin; NRBC, nucleated erythrocyte.

lysates from individual bursts (6) indicate that only between 17 and 80% of bursts contain HbF.

We have recently developed microdensitometric techniques for the quantitation of HbF or adult hemoglobin (HbA) in individual erythrocytes, reticulocytes, and nucleated erythrocytes (NRBC) (7). Application of the technique to NRBC derived from peripheral blood bursts should answer the following questions. Are NRBC containing HbF (FNRBC) present in all bursts? Is increased HbF production in culture due to increased production of FNRBC, increased amounts of HbF/FNRBC, or both? Is the production of F cells in culture regulated independently from the amount of HbF/cell as noted in vivo (3)? Answers to these questions should clarify at the cellular level the similarities and differences between HbF production in vivo and in culture.

METHODS

Erythropoietic cell culture. Mononuclear cells were isolated from heparinized peripheral blood of normal adult volunteers using modifications (8) of the Ficoll-Isopaque technique described by Boyum (9). Cell cultures were performed as previously reported (8) using slight modifications of the methylcellulose assay developed by Iscove et al. (10). In all cultures 1.0 IU/ml of Step III preparation of sheep plasma erythropoietin (Connaught Laboratories, Willowdale, Ontario, Canada) was used. On culture day 14, cells were harvested by diluting the culture mixture with isotonic phosphate-buffered saline, pH 7.4, and then transferred into a 5-ml plastic centrifuge tube. In studies in which individual bursts or subcolonies from one burst were analyzed, each burst or subcolony was lifted separately from the culture dish using a 10- μ l Eppendorf pipette and placed in microcentrifuge tubes containing ~250 μ l of 0.8% methylcellulose.

Single cell assay. Samples containing pooled bursts, an individual burst, or an individual subcolony were diluted with 1 ml of phosphate-buffered saline and centrifuged at 8,000 g for 3 min. The pellet of NRBC was washed three times with 1 ml phosphate-buffered saline and centrifuged at 400 g for 30 s. Washed NRBC were suspended in 20 to 40 μ l of isotonic borate saline, pH 8.3 (2). Aggregates of NRBC were disrupted by gentle tapping of the side of the tube. This cell suspension was divided into two equal aliquots. Each aliquot was added to a mixture containing 25 μ l 2.5% agarose, 5 μ l new methylene blue, and 25 μ l of antibody to either human HbF or HbA. The suspension of NRBC in agarose gel was placed on microscopic slides (2), and following lysis of the NRBC with lysolecithin those cells with a HbF-anti-HbF or HbA-anti-HbA pericellular immunoprecipitate reaction were enumerated (2, 7).

At least 3×10^6 cells were recovered after wash from pooled burst samples. In individual burst samples, between 1,000 and 3,000 cells were recovered for each assay. Between 50 and 250 cells were present in individual subcolonies. In all assays, between 85 and 95% of the NRBC reacted with anti-HbA (mean, $90.1 \pm 3.0\%$). In pooled burst samples, the percentage of NRBC reacting with anti-HbA corresponded exactly to the percentage of washed NRBC staining with benzidine, indicating that all NRBC assayed contained HbA and that 5–15% NRBC may have lysed before or during the washing procedure. In view of this, the percentage of FNRBC was expressed as the percentage of cells reacting with anti-HbF divided by the percentage of cells reacting with anti-HbA.

Using a recently devised microdensitometric method (7), the picograms of HbF or HbA in individual NRBC were quantitated. For each sample at least 100 NRBC containing HbF or HbA were examined and values were expressed as picograms Hb/NRBC (mean \pm SD).

RESULTS

Pooled bursts. Analysis of pooled bursts from each of 11 normal subjects demonstrated that the mean percentage of FNRBC found in culture is 13.5-fold greater than the mean percentage of F cells produced in vivo (Table I). This increase in F-cell production beyond the normal in vivo range, 0.5 to 7.5% (11), is similar to the previously reported 10- to 18-fold increase in lysate HbF synthesis in culture (12, 13). There was no correlation between peripheral blood F-cell levels and percentage of FNRBC produced in culture ($P = 0.50$).²

In a separate study we noted that HbF production in vivo is essentially complete early in normal erythroid development, i.e., the picograms of HbF in mature peripheral blood erythrocytes is not significantly different from HbF found in nucleated erythroid precursors in bone marrow aspirates (7). Therefore a comparison in normal adults between mean cellular HbF content in NRBC from erythropoietic bursts and mean HbF levels in peripheral blood erythrocytes should determine whether or not the amount of HbF/NRBC in culture is increased beyond in vivo NRBC levels. In five subjects (Table I, subjects 1, 2, 3, 4, and 7), the mean picograms HbF/NRBC, 5.3 ± 0.6 pg (mean \pm SE), was 23% greater than the mean F-cell levels ($P = 0.05$). In bone marrow aspirates from five normal subjects previously studied, HbF/NRBC was 4.3 ± 0.2 pg and HbA/NRBC was 22.0 ± 0.7 pg (7). These in vivo values are 20% less for HbF ($P = 0.04$) and 25% less for HbA ($P > 0.01$) than the mean picograms HbF/NRBC and picograms HbA/NRBC values found in culture for all 10 subjects in Table I. Since there is little difference in the relative increase in the cellular levels of HbF and HbA, the marked increase in HbF production in culture is due primarily to the 13-fold increase in the numbers of cells containing HbF. Among the 10 subjects studied there was no correlation in culture between percentage of FNRBC and picograms of HbF/NRBC ($P = 0.1$).

Individual bursts. In 108 individual bursts from three subjects and 58 individual bursts from subject 1, FNRBC were found in every burst. In subject 1 the mean percentage of FNRBC per burst was 48.7%, a value that closely parallels the 43.7% FNRBC value derived from pooled bursts from the same culture (see Fig. 1 and Table I, subject 1b). In subject 1 the

² All probability statistics in this paper were performed using the Mann-Whitney or Spearman's Rank method when comparing samples with unequal variances, and the Student *t* test when comparing samples with equal variances.

TABLE I
Single-Cell Analysis of Pooled 14-d Bursts from Each of 11 Normal Subjects

Subject	Percentage of F cells		Picograms Hb/cell		
	Burst FNRBC %	Peripheral blood F cell %	Burst picograms HbF/NRBC	Peripheral blood picograms HbF/F Cell	Burst picograms HbA/NRBC
1 a	40.8±6.0*	—	4.6±0.3	—	21.8±1.9
b	43.7±5.0	—	5.7±1.2	—	22.3±3.2
c	52.8±7.0	0.9±0.2	4.8±0.4	4.3±1.0	23.0±5.0
2 a	49.8±12.1	—	7.7±2.0	—	30.4±1.8
b	51.1±6.0	2.5±0.3	—	4.2±1.2	—
3	32.9±8.1	1.0±0.2	4.8±0.5	4.3±0.8	24.0±3.0
4	11.9±2.8	2.0±1.7	5.1±1.6	4.6±1.2	29.1±4.0
5	30.1±4.7	1.0±0.5	4.0±0.8	—	22.3±4.8
6	31.3±4.0	6.3±0.7	5.9±2.7	—	35.1±3.9
7	24.4±3.5	1.4±0.6	4.1±1.0	4.1±0.7	26.4±4.2
8	44.0±2.7	2.0±1.0	5.4±3.0	—	34.2±5.0
9	19.4±3.7	3.4±0.5	5.2±2.8	—	29.4±3.8
10	24.0±4.2	2.1±0.5	4.8±1.5	—	23.5±4.2
11	12.0±3.5	2.0±1.0	—	—	—
Mean±SE	29.6±3.9	2.2±0.5	5.2±0.4	4.3±0.1	27.7±1.5

* Mean±SD.

percentage of FNRBC per individual burst seems to be normally distributed between 5 and 95% (Fig. 1). This observation agrees with the broad normal distribution of the amount of HbF per burst previously reported by Kidoguchi et al. (4).

Aliquots from 22 of the 58 individual bursts analyzed in subject 1 were assayed for mean picograms HbF/NRBC and picograms HbA/NRBC (inset, Fig. 1). The

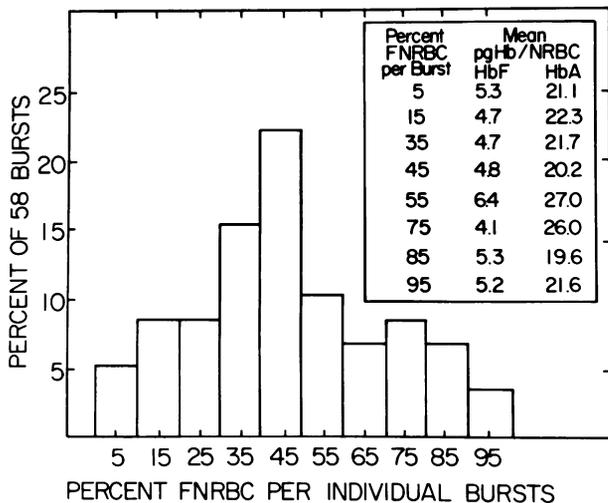


FIGURE 1 Relative frequency of 58 individual bursts from a normal subject in relation to the percentage of FNRBC per individual burst. Inset represents mean picograms HbF/NRBC and mean picograms HbA/NRBC for those bursts whose percentage of FNRBC are represented by the bars in the horizontal axis of the figure. Bursts are from subject 1 in Table I.

average picograms HbF/NRBC in individual bursts ranged from 4.1 to 7.6 pg (mean±SE for 22 bursts is 5.0±0.2 pg). Just as noted in pooled burst analysis, there was no correlation between the mean picograms HbF/NRBC and the percentage of FNRBC in individual bursts ($P = 0.73$). In addition, no correlation could be found in individual bursts between mean picograms HbA/NRBC and either percentage of FNRBC ($P = 0.57$) or mean picograms HbF/NRBC ($P = 0.20$). It therefore appears that neither the amount of HbF/NRBC nor the percentage of FNRBC is a function of how much HbA is produced in individual bursts.

Subcolonies. Subcolonies from one burst were examined in two subjects (Table II). If commitment to the formation of HbF was complete before subcolony formation, we would have expected to see subcolonies with either no FNRBC or 100% FNRBC. However, all subcolonies contained some FNRBC and none had 100%. In subject 8 a subcolony with 3.0% FNRBC and a subcolony with 81% FNRBC had essentially the same number of total cells (see legend, Table II). This observation excludes the possibility that the variation in the percentage of FNRBC among subcolonies is due to fusion of subcolonies containing no or 100% FNRBC. This, in turn, suggests that commitment to HbF production is ongoing throughout the life span of the burst.

DISCUSSION

In a previous report we noted that acquired elevation of HbF in vivo was a general phenomenon asso-

TABLE II

Percent Nucleated Erythrocytes Containing HbF in Individual Subcolonies from a Single Burst in Each of Two Subjects

Subject	Percent FNRBC/subcolony	Subject	Percent FNRBC/subcolony
5	68.0±5.6	8	3.0±1.4*
	69.5±1.9		20.7±1.2
	33.7±8.7		22.0±3.7
	52.4±4.2		51.8±3.9
	29.3±8.3		81.0±3.6*
	35.3±0.5		33.0±1.4
	49.3±7.1		

* The subcolony with 3.0 and 81.0 percent FNRBC contained 209 and 190 total NRBC, respectively.

ciated with acute erythropoietic expansion (14). Increased F-cell number was uniformly associated with acute erythroid stimulation while the amount of HbF/cell did not necessarily change (2, 14). The present observations suggest that increased HbF production in culture is primarily due to a 6- to 50-fold increase in F-cell number while HbF and HbA per NRBC were equally increased by ~20% over in vivo levels.

All bursts examined contained FNRBC in varying percentages. This finding agrees with isoelectric focusing of radiolabeled hemoglobin from individual bursts (4) but is discrepant from previously reported immunologic assays of HbF in single bursts (5, 6). These immunologic assays either analyzed aggregates of cells within a burst with fluorescent anti-HbF (5), or performed radioimmunoassays of lysate from individual bursts (6). It is possible that these techniques are not sensitive enough to detect <5% FNRBC with only 4 pg HbF/NRBC or that culture conditions vary significantly between laboratories.

Our results also differ somewhat from the finding of Papayannopoulou et al. (5) that the ability to produce HbF in culture is inversely related to the degree of differentiation of the erythroid precursor, i.e., HbF levels are higher in colonies derived from earlier erythroid progenitor vs. HbF level in colonies derived from late erythroid precursors (5). We find that the variation in the percentage of FNRBC between individual bursts from the same culture is similar to the variation in the percentage of FNRBC between subcolonies from a single burst. This suggests that commitment to HbF production is ongoing throughout the 14 d of burst formation and is not irrevocably programmed at a single early state of erythroid development.

ACKNOWLEDGMENT

This investigation was supported in part by National Institutes of Health grant HL20913 and the Veterans Administration.

REFERENCES

- Papayannopoulou, T., M. Brice, and G. Stamatoyannopoulos. 1976. Stimulation of fetal hemoglobin synthesis in bone marrow cultures from adult individuals. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 2033-2037.
- Dover, G. J., S. H. Boyer, and W. R. Bell. 1978. Microscopic method for assaying F cell production: illustrative changes during infancy and aplastic anemia. *Blood.* **52**: 664-672.
- Dover, G. J., S. H. Boyer, S. Charache, and K. Heintzelman. 1978. Individual variation in the production and survival of F cells in sickle cell disease. *N. Engl. J. Med.* **299**: 1428-1435.
- Kidoguchi, K., M. Ogawa, and J. D. Karam. 1979. Hemoglobin biosynthesis in individual erythropoietic bursts in culture. Studies of adult peripheral blood. *J. Clin. Invest.* **63**: 804-806.
- Papayannopoulou, T., T. Kalmantis, and G. Stamatoyannopoulos. 1979. Cellular regulation of hemoglobin switching: evidence for inverse relationship between fetal hemoglobin synthesis and degree of maturity of human erythroid cells. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 6420-6424.
- Fausner, A. A., and H. A. Messner. 1979. Fetal hemoglobin mixed hemopoietic colonies (CFU-GEMM), erythroid bursts (BFU-E), and erythroid subcolonies (CFU-E): assessment by radioimmune assay and immunofluorescence. *Blood.* **54**: 1384-1394.
- Dover, G. J., and S. H. Boyer. 1980. Quantitation of hemoglobins within individual red cells: asynchronous biosynthesis of fetal and adult hemoglobin during erythroid maturation in normal subjects. *Blood.* **56**: In press.
- Ogawa, M., O. C., Grush, R. F. O'Dell, H. Hara, and M. D. MacEachern. 1977. Circulating erythropoietic precursors in culture: characterizations in normal men and patients with hemoglobinopathies. *Blood.* **50**: 1081-1092.
- Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97): 77-89.
- Iscove, N. N., F. Sieber, and K. H. Winterhalter. 1974. Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose concanavalin A. *J. Cell. Physiol.* **83**: 309-320.
- Boyer, S. H., L. Margolet, M. L. Boyer, T. H. Huisman, W. A. Schroeder, W. G. Wood, D. J. Weatherall, J. B. Clegg, and R. Cartner. 1977. Inheritance of F cell frequency in heterocellular hereditary persistence of fetal hemoglobin: an example of allelic exclusion. *Am. J. Hum. Genet.* **29**: 256-271.
- Papayannopoulou, T., B. NaKamoto, J. Buckley, S. Kurachi, P. E. Nute, and G. Stamatoyannopoulos. 1978. Erythroid progenitors circulating in the blood of adult individuals produce fetal hemoglobin in culture. *Science (Wash. D. C.)* **199**: 1349-1350.
- Kidoguchi, K., M. Ogawa, J. D. Karam, and A. G. Martin. 1978. Augmentation of fetal hemoglobin (HbF) synthesis in culture by human erythropoietic precursors in the marrow and peripheral blood: studies in sickle cell anemia and non-hemoglobinopathic adults. *Blood.* **52**: 1115-1124.
- Dover, G. J., S. H. Boyer, and W. H. Zinkham. 1979. Production of erythrocytes that contain fetal hemoglobin in anemia. Transient in vivo changes. *J. Clin. Invest.* **63**: 173-176.