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J Clin Invest. 1979;63(4):804-806. https://doi.org/10.1172/JCI109366.

Research Article

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Hemoglobin Biosynthesis in Individual Erythropoietic Bursts in Culture

STUDIES OF ADULT PERIPHERAL BLOOD

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ABSTRACT By using a methylcellulose clonal assay, we cultured peripheral blood erythropoietic precursors from a patient with sickle cell anemia, a patient with sickle cell hemoglobin C disease, and a normal volunteer. We then analyzed the synthetic rates of adult and fetal hemoglobins (Hb) in individual erythropoietic bursts. Hb were labeled with ^{14}C-amino acids in culture, separated by slab gel isoelectric focusing techniques, and quantitated by fluorographic methods. All bursts exhibited both fetal and adult Hb in varying ratios. Frequency distributions of the individual bursts differing in percentage of HbF biosynthesis approached normal distributions. Further stimulation of HbF synthesis by higher erythropoietin in culture was associated with increased HbF biosynthesis in individual bursts. Augmentation of human HbF synthesis in culture appears to be controlled by qualitative intracellular changes rather than by changes in cellular population.

INTRODUCTION

Elucidation, and possibly manipulation, of the mechanisms controlling globin gene expression is being actively pursued for potential application in the treatment of patients with various hemoglobinopathies (1). Observations in a number of clinical conditions associated with intense erythropoiesis suggest that adult human marrow cells are capable of reactivating synthesis of fetal hemoglobin (HbF)[1] (1). It has also been noted that adult marrow cells are capable of synthesizing an increased amount of HbF when cultured in the presence of erythropoietin. By using a clonal cell culture assay, Papayannopoulou et al. (2) established fundamental concepts regarding Hb biosynthetic capabilities of adult marrow erythropoietic precursors. They reported that HbF biosynthetic capabilities of human erythropoietic precursors are inversely related to their maturational stages and that some of the early erythropoietic precursors termed erythropoietic burst-forming units (BFU-E) are capable of expressing both fetal and adult globin genes in their differentiated progenies. More recently, this group of investigators reported concordance of gamma mRNA content and gamma-chain synthesis in culture using hybridization techniques with complementary DNA probes (3). Their results strongly indicated that Hb switching in culture is regulated at transcriptional levels. We have shown that Hb biosynthetic capabilities of adult BFU-E in peripheral blood are identical to that of BFU-E population in the marrow (4). With fluorographic methods, we have now succeeded in characterization of Hb biosynthesis in individual erythropoietic bursts in culture. Here we report studies on peripheral blood BFU-E from an adult patient with sickle cell anemia, a patient with sickle cell HbC disease (SC disease) and a healthy adult volunteer.

METHODS

Erythropoietic cell cultures. Mononuclear cells were harvested from the blood by using slight modifications (5) of the Ficoll-Isopaque technique (Ficoll, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; Isopaque, Winthrop Laboratories, New York) described by Boyum (6). Cell culture was carried out for

A portion of this study was presented at the 21st Annual Meeting of the American Society of Hematology and has been published in abstract form in 1978: *Blood.* **52**(Suppl. 1): 206.

Dr. Ogawa is a Leukemia Society of America Scholar. Dr. Karam is the recipient of Research Career Development Award KO4 GM70725 from the U. S. Public Health Service. Address reprint requests to Dr. Ogawa at the Veterans Administration Medical Center, 109 Bee St., Charleston, S. C. 29403.

Received for publication 8 January 1979 and in revised form 5 February 1979.

[1] *Abbreviations used in this paper*: BFU-E, erythropoietic burst-forming units; Ep, erythropoietin; Hb, hemoglobin; SC disease, sickle cell hemoglobin C disease.

J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.* · *0021-9738/79/04/0804/03* $1.00
Volume 63 April 1979 804–806

14 d with slight modifications (5) of the methylcellulose assay developed by Iscove et al. (7). We used human urinary erythropoietin (Ep) with a 40-U/mg sp act, which was supplied by the Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute, Bethesda, Md. Cells from the patient with sickle cell anemia were cultured simultaneously in the presence of Ep concentrations of 0.5 and 1.0 U/ml. Cells from the normal volunteer and the patient with SC disease were cultured in 1.0 U/ml Ep concentration.

Analysis of Hb synthesis in individual bursts in culture. Measurements of rates of Hb synthesis were carried out during the last 2 d of culture (4). On day 12 of incubation, 2 μCi of uniformly ^{14}C-labeled amino acid mixture (NEC-445, New England Nuclear, Boston, Mass.) in 0.3 ml phosphate-buffered saline was layered over a dish containing 1.0 ml of tissue culture mixture. On day 14 of incubation, individual bursts were identified by viewing through a magnifier and randomly lifted from the methylcellulose medium by using a 10-μl Eppendorf pipette (Brinkmann Instruments, Inc., Westbury, N. Y.). The samples were individually placed in microcentrifuge tubes containing 200 μl phosphate-buffered saline and 0.5 μl of packed erythrocytes prepared from the original blood samples. After centrifugation, the cell pellets were frozen at −70°C overnight. The frozen cell pellets were thawed at room temperature and lysed by the addition of a solution containing 4 μl of 0.01 M KCN and 1 μl of 10% Nonidet P-40 (Shell Chemical Co., New York). Hemolysates of individual bursts were subjected to isoelectric focusing, which was carried out on an LKB Multiphor apparatus (LKB Instruments, Inc., Rockville, Md.) as described previously (4). After completion of the run, the gels were fixed in 15% TCA, equilibrated with dimethylsulfoxide, impregnated with 2,5-diphenyloxazole in the presence of dimethylsulfoxide, and soaked in water (8). Gels were dried and then for one week were placed in contact with Kodak RP "X-Omat" film (Eastman Kodak Co., Rochester, N. Y.) that had been previously exposed to a brief flash of light using an electronic photographic flash unit (Vivitar 283; Vivitar Corp., Div. of Ponder & Best Inc., Santa Monica, Calif.). The densities of fluorogram bands were determined from densitometric tracings that were carried out on a Joyce/Loebl microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England). The prior exposure to a flash of light had raised the level of background fog absorbance of the film by 0.15 OD and had established a linear relationship between radioactivity of the sample and density of the fluorogram band (8).

For analysis of the frequency distributions of HbF biosynthesis, each burst was ranked according to its percentage of HbF, and the frequency probability (P_i) was calculated on the basis of $P_i = (O_i - 0.5)/n$, where O_i and n represent the rank of the individual sample and total number of the samples, respectively. Empirical probits of samples directly transformed from $P_i \times 100$ (9) were then plotted against their HbF biosynthesis.

RESULTS

We examined Hb biosynthesis in a total of 69 erythropoietic bursts from the cultures of peripheral blood mononuclear cells obtained from a patient with sickle cell anemia. Cells were plated in the presence of 0.5 and 1.0 U/ml Ep concentrations. All bursts contained HbS and HbF. The ratio of fetal to adult Hb, however, varied among individual bursts as is shown in a representative portion of a fluorogram of the sickle cell anemia patient (Fig. 1). Cumulative frequency distributions of

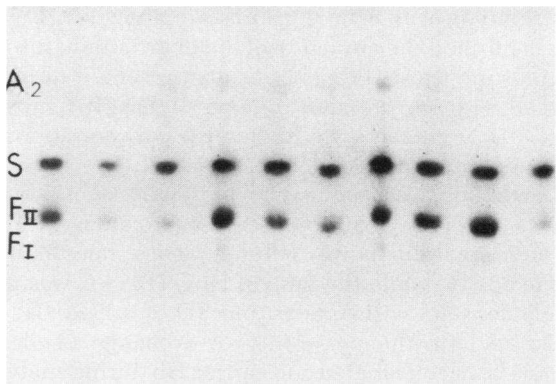

FIGURE 1 Portion of a fluorogram from the sickle cell anemia patient showing separation by isoelectric focusing of ^{14}C-labeled Hb in individual bursts.

individual bursts varying in percentage of HbF biosynthesis approached normal distributions. The mean and SD of the distributions were 39±10.1 and 51.2 ±15.7% for the low (0.5 U/ml) and high (1.0 U/ml) Ep groups, respectively. Examinations of HbF biosynthesis in 35 bursts from the normal donor also revealed a normal frequency distribution with a mean and SD of 31.2±13.4%. Analyses of the goodness of fit to a normal

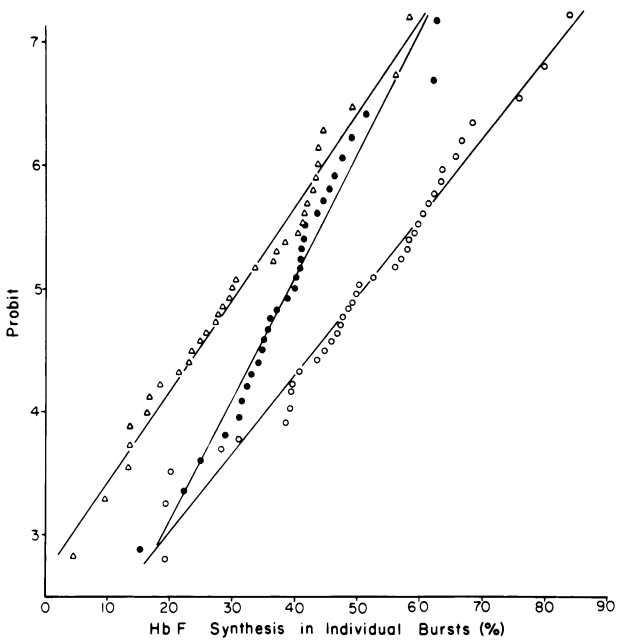

FIGURE 2 Goodness of fit to normal distributions of the samples demonstrating normal deviates of individual bursts. Symbols represent empirical probits of individual bursts (△, normal donor; ●, sickle cell anemia cultured with 0.5 U/ml Ep; ○, same sample with 1.0 U/ml Ep). The lines represent expected normal values (9). Distributions of the two sickle cell groups are significantly different ($P < 0.001$) by both the t test and by the Mann and Whitney test.

distribution of the three groups are shown in Fig. 2. We confirmed the normality of the distributions also by D'Agostino's test (10). The results presented in Fig. 2 agreed with our previous finding (4) that HbF biosynthesis is augmented by higher Ep concentrations in culture.

In addition, we examined Hb biosynthesis in 15 bursts from a patient with SC disease. The percentage of HbF in individual bursts was heterogeneous, ranging from 4.0 to 35.7%, while the ratio of HbC/HbS+C was relatively constant with a mean and SD of 0.57±0.04.

To exclude the possibility of exchange of globin chains between labeled and carrier Hb during analysis, sickle cell erythrocytes were added to labeled cultures of normal peripheral blood mononuclear cells and the samples were processed for fluorography. There was no radioactive peak in the area corresponding to HbS; demonstrating that under the conditions we employed, there was no significant globin chain exchange between labeled and unlabeled Hb.

DISCUSSION

Two models may be proposed as cellular mechanisms for the augmentation of HbF synthesis in culture of adult erythropoietic precursors. The first model assigns particular Hb types to specific populations of erythroid precursors and switching to differential selection of cell populations in culture. The second model hypothesizes qualitative changes in a single stem cell line. Weatherall et al. (11) summarized clinical evidence and predicted that HbF reactivation in adults is controlled by the former mechanism. According to this hypothesis, the presence of BFU-E committed to synthesis of either adult or fetal Hb is expected.

To gain an insight into the mechanism of Hb switching, we examined Hb biosynthesis in individual bursts from adult subjects. We developed a fluorographic microassay for quantitation of Hb biosynthesis in culture. The validity of this technique was provided by the studies of a patient with SC disease. While the ratio of fetal to adult Hb varied widely, the ratio of HbC/HbS+C did not fluctuate significantly in individual bursts.

All of the erythropoietic bursts examined with this method contained both adult and fetal Hb in varying ratios. This is in contrast to a bimodal distribution which would have been predicted by the first model. Furthermore, augmentation of HbF biosynthesis by a higher Ep dose in culture resulted from an increment in the HbF production in individual bursts as illustrated by the shift of the distribution in Fig. 2. Appearance of a separate BFU-E population would have resulted in a deviation from a normal distribution. Although we cannot exclude the possibility of two closely overlapping populations, these results strongly support the second model. The closeness to normal distributions of the frequency distributions of bursts differing in HbF synthetic capabilities further suggests that the Hb switching of human BFU-E in culture is a random occurrence. Augmentation of HbF synthesis in patients with beta-chain abnormalities may need to be approached by studies of intracellular mechanisms rather than by selection of cellular populations.

ACKNOWLEDGMENTS

We thank Mrs. Juraee S. McNeil, Ms. Alyce G. Martin, Mrs. Lobelia A. Avila, Mr. Earl L. Alston, and Mr. Clinton O. Fullwood for their excellent technical assistance. We also thank Ms. P. Linda Skipper for her help in preparation of this manuscript.

This investigation was supported by National Institutes of Health grant HL20913 and by Veterans Administration Basic Institutional Funds.

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