Hb F Production in Endogenous Colonies of Polycythemia Vera

By Th. Papayannopoulou, J. Buckley, B. Nakamoto, S. Kurachi, P. E. Nute, and G. Stamatoyannopoulos

Fetal hemoglobin was studied in endogenous colonies produced in plasma clot and methylcellulose cultures of circulating progenitors from patients with polycythemia vera (PV). Analysis of globin chain synthesis showed that γ chains constituted from 13% to 42% of the non-α chains produced in cultured cells, whereas from 27% to over 50% of the endogenous colonies contained Hb F, as indicated by the fluorescent antibody probe. Since the endogenous colonies in PV cultures originate from the abnormal PV clone, the findings provide direct evidence that a single pluripotent stem cell can have committed progeny that differ in their expressions of the Hb F production program.

POLYCYTHEMIA VERA (PV) is a myeloproliferative disorder of apparently clonal origin arising in a pluripotent stem cell. In females with this condition who are also heterozygous for the A and B alleles of the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD), circulating red cells, granulocytes, monocytes, and platelets all contain the same one of the two G6PD isozymes, thus indicating that all these formed elements of blood originate from a single pluripotent stem cell, i.e., the one bearing the PV abnormality.[1] One of the in vitro characteristics of PV is the appearance of erythroid colonies (endogenous E colonies) in cultures from which exogenously added erythropoietin has been omitted.[2] When endogenous colonies have been studied in cultures from female G6PD AB heterozygotes, all have shown only one of the two G6PD isozymes, the same as that present in the patient's blood.[3] These data imply that the erythroid progenitors capable of giving rise to endogenous colonies are descendants of a single clone, the PV clone. The previously mentioned culture characteristics of the PV cells, namely the presence under certain conditions of a population of colonies representing a single pluripotent stem cell, provide the opportunity for in vitro testing of the hypothesis that the Hb phenotype of the maturing erythron is determined at the level of the pluripotent stem cell.[4] The results of such a study are described in this report.

MATERIALS AND METHODS

Eleven patients with PV were studied. They all fulfilled the diagnostic criteria for PV as outlined by the PV study group.[5] All had been treated with phlebotomy in the past, whereas 6 of the 11 had, in addition, had ^{32}P and myelotoxic therapy. Routine hematologic studies and measurements of Hb F levels and F-cell counts[6] were carried out at the time of study.

From the Divisions of Hematology and Medical Genetics, Department of Medicine, University of Washington, Seattle, Wash.

Submitted July 26, 1978; accepted November 9, 1978.

Supported by Grants HL-20899, HL-20961, and GM-15253 from the National Institutes of Health.

Address reprint requests to Thalia Papayannopoulou, M.D., Division of Hematology, Department of Medicine, University of Washington, Seattle, Wash. 98195.

Cell Cultures

Peripheral blood mononuclear cells were separated and processed as previously described.[7] Concentrations of inoculated cells varied from 0.25×10^6 to 1×10^6 cells/ml of medium. Cultures were grown in plasma clots or methylcellulose media;[7,8] erythropoietin was omitted from the culture media when indicated. For the erythropoietin response experiments, partially purified sheep plasma preparations (step III, Connaught Medical Research Laboratories, Toronto, Canada) or human urinary erythropoietin were used. Erythroid colony growth was monitored by counting the hemoglobinized colonies after benzidine staining of plasma clots. Colony counts were performed serially from culture day 6 to day 17. For identification of Hb F and Hb A production in situ, plasma clots were labeled with anti-Hb-F antibodies conjugated to fluorescein isothiocyanate (anti-Hb-F-FITC) and anti-Hb-A antibodies conjugated to rhodamine, as previously described.[9]

Hemoglobin Synthesis

The newly synthesized globin chains were measured in erythroid colonies from either plasma clot plates or methylcellulose plates. Labeling of the colonies with the radioactive amino acid was carried out at culture days 10–12, depending on the colony growth. Plates with fibrin clots were labeled in situ for a period of 24 hr using ^{35}S-methionine (specific activity 700–900 Ci/mmole); at the end of the labeling period they were processed as previously described.[8] With methylcellulose cultures an alternative labeling method was used: orange-red colonies were plucked from the plates by the use of ultrathin glass pipettes, washed two to three times in medium, and incubated overnight under gentle agitation at 37°C in a methionine-free incubation medium containing ^{35}S-methionine, 19 nonradioactive amino acids, and ferrous ammonium sulfate. The cells were washed in saline and lysed, and the lysates (after addition of ^{3}H-methionine-labeled Hb F and Hb A as carriers) were purified by gel filtration.[8] Hemoglobins eluted from gel filtration columns were converted to globin, and their constituent chains were isolated on carboxymethylcellulose columns. The material eluting with ^{3}H-γ and ^{3}H-β carrier was digested with trypsin. Contamination of γ-chain peaks by pre-β chains was corrected for by measuring the proportions of ^{35}S radioactivity in the positions occupied by tryptic peptides β^5 and γ^5 after high-voltage paper electrophoresis of the tryptic digests.[8]

RESULTS

Endogenous Erythroid Colony Formation by Circulating PV Progenitors

Erythroid colonies were formed in the absence of added erythropoietin in cultures of cells from 7 of the 11 subjects. Endogenous colonies appear somewhat earlier than blood BFU-E-derived* colonies from normal controls; this is illustrated in Fig. 1, in which the normalized rates of appearance of erythroid colonies in peripheral blood cultures from 9 normal controls and from 2 patients with PV are compared. Sixty percent to 70% (of the peak growth observed) of these endogenous colonies appeared by day 8 in culture, and the majority were hemoglobinized; in contrast, only 10%–15% of the maximum number of colonies from normal blood BFU-E control cultures were present at that time, and the majority were not hemoglobinized. Plating efficiencies (number of colonies at peak colony formation) ranged from 16 to 802 endogenous colonies per 10^5 inoculated mononuclear cells (Table 1). Several of the endogenous colonies assumed a clustered or erythroid burst formation, whereas most of them appeared in a scattered fashion (Fig. 2). Individual colony sizes ranged from 8 to 400 cells.

Addition of erythropoietin resulted in an increase in the number and size of BFU-E-derived colonies. Although the maximum numbers of erythroid colonies were obtained when 2.0–5.0 IU of erythropoietin per milliliter of medium were

*BFU-E = burst-forming unit-erythroid.

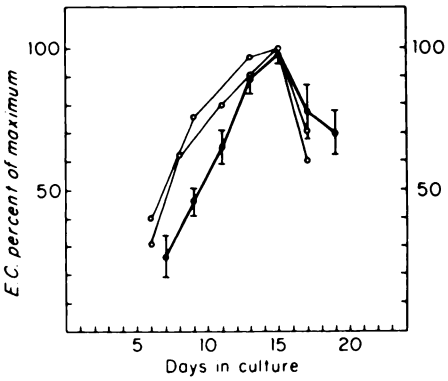

Fig. 1. Rate of appearance of erythroid colonies in cultures of blood mononuclear cells from 2 PV patients (cases 3 and 4, Table 1) is compared with rate of appearance of colonies in cultures of mononuclear cells from healthy adults: white circles indicate endogenous PV colonies; black circles indicate erythroid colonies in normal control cultures; the latter cultures were done in the presence of 2.0 units of erythropoietin, and the curve represents the sum of nine experiments. Data are expressed as percentage of maximum number of observed colonies in order to adjust for the significant difference in plating efficiency between samples. Due to difficulties in accurate counting of erythroid bursts in the flattened plasma clot cultures, the numbers of individual colonies, not erythroid bursts, are used in the evaluations presented throughout this article.

Table 1. Cloning Efficiency for Endogenous Colonies and Relative Numbers of Hb-F-Containing Endogenous Colonies in 7 Patients With PV

Patient Number*	F Cells In Vivo (%)	Number of Endogenous Colonies† (per 10^5)	F Endogenous Colonies‡ (%)
1	2.6	28	29.6
2	2.7	506	41.2
3a	1.9	76	52.7
3b	1.9	206	44.4
3c	1.4	237	40.4
4a	10.4	371	52.9
4b	7.5	802	33.0
5	3.7	33	27.3
6	1.9	61	—
7	9.5	16	—

*Letters a, b, and c denote separate experiments done from 2 to 8 mo apart.

†Counts (culture days 10 to 15) refer to individual, partially, or completely separated benzidine-positive erythroid colonies; counts of erythroid bursts are not provided, since objective measurements of erythroid bursts in the flattened plasma clots were not always possible.

‡Includes colonies in which both Hb F and Hb A were detectable as well as colonies in which only Hb F was detectable after reaction with the fluorescent antibodies.

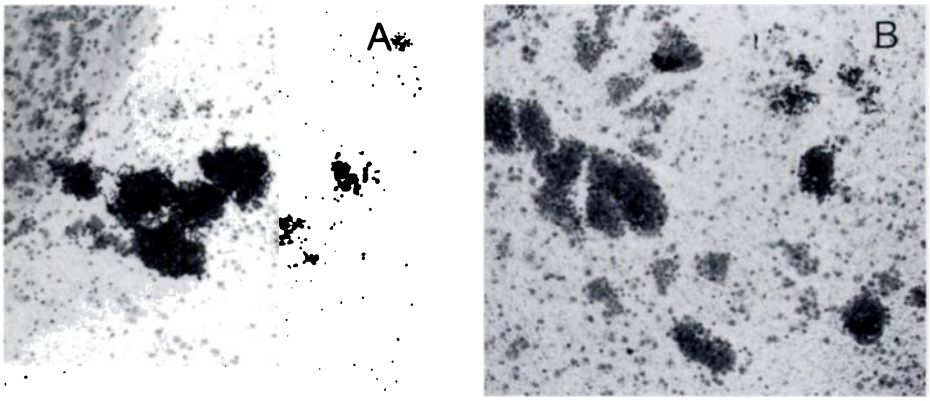

Fig. 2. Morphology of endogenous colonies from circulating erythroid progenitors of PV. Several of the colonies are arranged in the form of erythroid bursts. (A) Erythroid colonies at culture day 14 (case 3, Table 1). (B) Colonies at culture day 10 (case 4, Table 1).

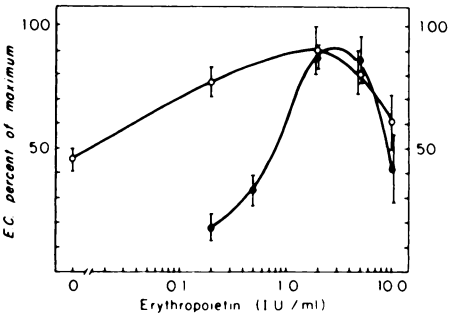

Fig. 3. Erythropoietin response curve of PV circulating erythroid progenitors is compared with that of circulating progenitors of adult healthy individuals. The PV data (white circles) are derived from the normalized values from five experiments and the control data (black circles) from cultures of 5 healthy adult volunteers. E.C. in the ordinate of the diagram denotes erythroid colonies.

added, there were significant responses to low levels of erythropoietin (i.e., 0.02 and 0.2 IU/ml). The erythropoietin response curve of the circulating PV precursors differed strikingly from that of normal controls (Fig. 3).

Fetal Hemoglobin Synthesis in Endogenous Colonies

The synthesis of Hb F and Hb A in endogenous colonies was examined at the day of peak colony formation by use of fluorescent antibody probes. Among the hemoglobinized endogenous colonies, some contained both Hb F and Hb A, whereas others contained only one of the two hemoglobins (Figs. 4 and 5). Frequencies of F-positive colonies (i.e., colonies showing only Hb F or both Hb F and Hb A) ranged from 27% to 53% (Table 1). Thus, by the immunofluorescent probe, abundant Hb F production appears to occur in clones that have grown in culture without added erythropoietin.

Supporting evidence for elevated Hb F production was obtained by studies of globin biosynthesis. All the patients had less than 2% Hb F in their blood as shown either by chemical determination or by biosynthetic measurement of incorporation of ^{35}S radioactivity into the γ and β chains synthesized in blood reticulocytes. F-cell levels were in the normal range, with the exception of 2 individuals in whom F-cell levels were slightly above the normal range[6] (Table 1). In cultures raised in the absence of added erythropoietin, a significant amount of ^{35}S radioactivity was incorporated into the γ subunits (Fig. 6A and Table 2). Synthesis of γ chains in

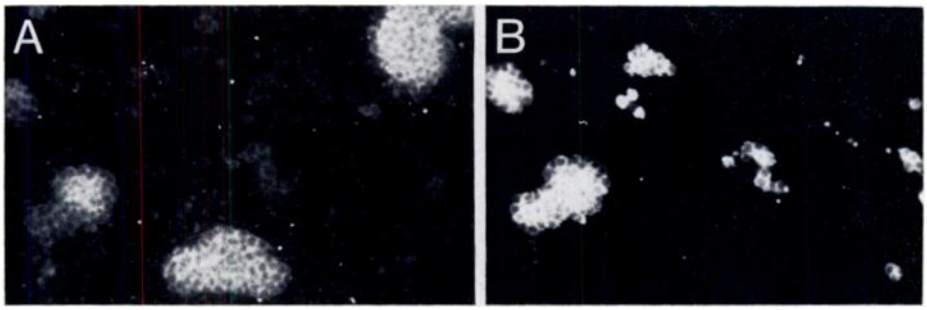

Fig. 4. Double immunofluorescent labeling of endogenous colonies using anti-Hb-F-FITC and anti-Hb-A antibodies conjugated to rhodamine (case 4, Table 1). (A) Colonies viewed through excitation beam for rhodamine. Three colonies appear positive for Hb A. (B) Same field viewed through the FITC excitation beam to test for presence of Hb F. Presence of Hb F cannot be detected in two colonies containing Hb A, whereas two other small colonies in this field appear to be positive only for Hb F.

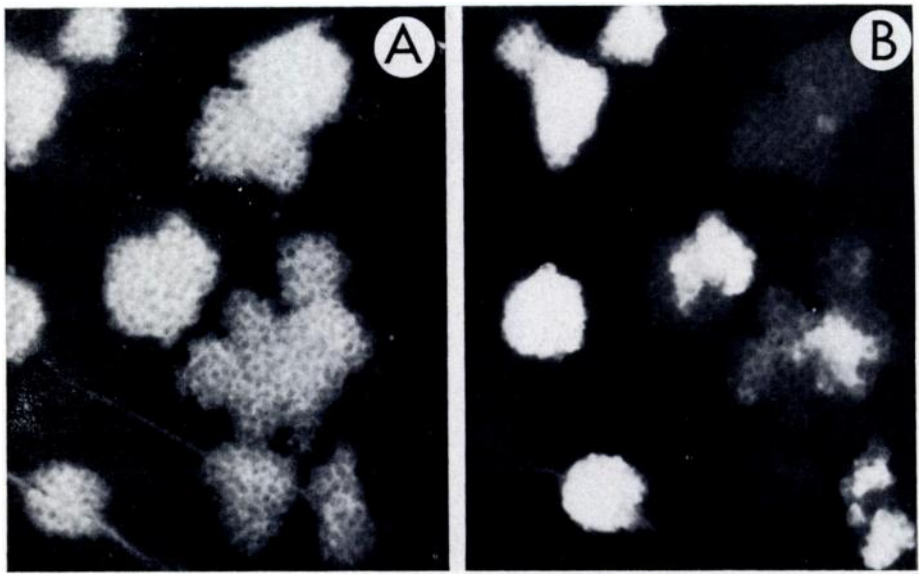

Fig. 5. Colonies (case 4, Table 1) were labeled as in Fig. 4 (A: labeling with anti-Hb-A rhodamine; B: labeling with anti-Hb-F-FITC) but they were grown in the presence of 0.5 IU of Epo. Addition of erythropoietin resulted in increases in both numbers and sizes of colonies. Note the presence of Hb A in all the colonies of panel A and the significant heterogeneity in the Hb F content of the same colonies shown in panel B.

Table 2. Globin Chain Synthesis in Cultured Circulating Progenitors From Patients With PV

Patient Number	Type of Culture*	Added Epo		Incorporation of ^{35}S Radioactivity‡ (CMP × 10^3)			γ-Chain Synthesis (% of non-α)
		Units/ml of medium	Source†	γ Chain	β Chain	α Chain	
3a	PC	0.0	Sheep	10.03	43.09	61.32	18.9
	MC	0.0	Sheep	2.22	10.46	11.27	17.5
	MC	2.0	Sheep	2.79	27.74	33.40	9.1
3b	MC	0.0	Sheep	12.23	52.52	90.00	18.9
	MC	0.5	Sheep	55.20	398.39	622.47	12.2
	MC	2.0	Sheep	27.47	580.86	860.66	4.5
	MC	5.0	Sheep	54.25	692.92	948.68	7.3
3c	PC	0.0	Sheep	12.76	53.93	80.07	19.1
	PC	0.5	Sheep	15.02	134.71	198.62	10.0
	PC	5.0	Sheep	5.94	132.88	209.50	4.3
4b	MC	0.0	Sheep	5.44	36.52	51.49	13.0
	MC	2.0	Sheep	8.92	49.36	78.44	15.3
	MC	5.0	Sheep	6.56	32.19	46.73	16.9
6	PC	0.0	Human	47.99	66.31	105.16	41.9
	PC	0.2	Human	23.34	85.49	111.00	21.5
	PC	2.0	Human	33.60	173.84	191.08	16.2
7	PC	1.0	Human	2.59	20.01	21.47	11.4
	PC	5.0	Human	1.24	12.82	19.54	8.8

*MC: methylcellulose was used as a supporting medium; PC: plasma clot media were used for culture.

†In experiments 6 and 7 human urinary erythropoietin was used (lot ARG-1-TALSL, specific activity 100 IU/mg protein). In experiments 3 and 4 the source of erythropoietin was sheep plasma (step III, Connaught Laboratories: lot 3017-4, specific activity 6.1 IU/mg protein for case 3; lot number 3018-15, specific activity 5.9 IU/mg protein for case 4).

‡Counts are corrected for the difference in methionine content (two per γ, one per β, and two per α chain).

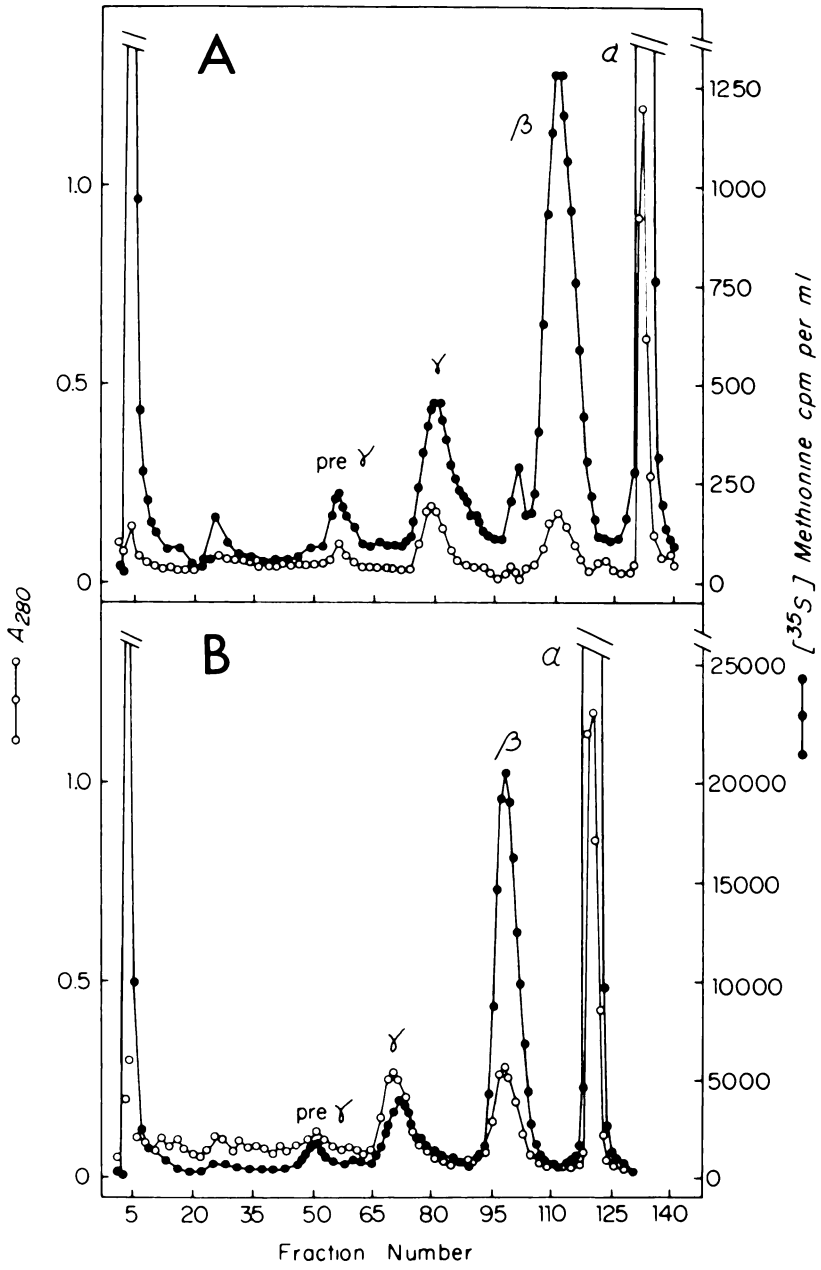

Fig. 6 Globin chain separation of culture lysates on carboxymethylcellulose columns. The lysates have been previously purified on G-100 Sephadex columns to remove nonhemoglobin contaminants. Incorporation of ^{35}S-methionine radioactivity in the globin chains from cultures raised (A) without added erythropoietin and (B) in the presence of 5.0 IU of erythropoietin.

endogenous colonies accounted for 13%–42% of the total non-α chains (Table 2). When γ-chain production at increasing levels of exogenously added erythropoietin was measured, a decrease in the proportion of γ chains was noted in three experiments, whereas no significant change was observed in one experiment (Table 2). The decrease in γ-chain synthesis was noted with human urinary erythropoietin and one lot of sheep plasma erythropoietin (Table 2).

DISCUSSION

The biosynthetic measurements in the experiments described in this report suggest that endogenous PV colonies produce a significant amount of γ chains. Immunofluorescent evaluation of the fixed preparations using fluorescent anti-Hb-F antibodies shows that among the endogenous erythroid colonies some with Hb F production and others with no detectable Hb F are present. These data provide information pertaining to two questions: (1) the pluripotent stem-cell determination of Hb F production and (2) the state of differentiation of the precursor cells that give rise to endogenous colonies in PV.

Pluripotent stem-cell determination of Hb F production. Fetal hemoglobin production in the adult has been explained on the basis of several mechanisms: a phenomenon related to erythroid stem-cell differentiation whereby the Hb-F-containing cells are the direct progeny of relatively primitive erythroid progenitors;[9] regulatory accidents affecting globin gene transcription;[11] derivation of F cells from separate lines of pluripotent stem cells programmed for Hb F formation.[4] According to the latter model proposed by Weatherall et al.,[4] at the perinatal period a proportion of pluripotent stem cells escapes the irreversible switch from F to A formation and gives rise to partially switched "pluripotent stem cells" that provide the F cells of the adult.

The postulate of separate pluripotent stem-cell origin of F cells has been tested with studies of cellular distribution of Hb F in patients with the clonal hemopoietic disorders chronic myelogenous leukemia and paroxysmal nocturnal hemoglobinuria. The findings are compatible with the interpretation that the abnormal clone proliferating in the patient's marrow can provide both F-cell progeny and A-cell progeny.[12,13] Direct evidence against separate pluripotent stem-cell determination of Hb F production is provided by the findings of the present report. As has been shown by the G6PD studies, endogenous colonies in PV cultures originate exclusively from the mutant PV clone.[3] If there are separate lines of pluripotent stem cells primed for either Hb A or Hb F formation,[4] the PV mutation must have occurred in either of the two types of stem cells, and therefore endogenous colonies should show either uniform presence or uniform absence of Hb F formation. Endogenous colonies derived from cells of patients without in vivo Hb F production (and, hence, with a mutation in the putative A pluripotent stem cell) should uniformly fail to show fetal hemoglobin. This prediction has not been fulfilled, since in our cultures brisk Hb F production was detected by biosynthetic means, and 30%–50% of the endogenous colonies contained Hb F as shown by the fluorescent probes. Thus it appears that either the postulate of separate pluripotent stem-cell lines does not apply in Hb F regulation or that Hb F synthesis in the clonal

hemopoietic disorders is regulated by a mechanism that differs from that in normal man.

State of differentiation of precursors of endogenous colonies. Studies of cultures of bone marrow cells of normal adults, using the immunofluorescent probes for identification of Hb A and Hb F in situ, have yielded findings compatible with the interpretation that the precursors giving rise to erythroid colonies with Hb F (F colonies) have the growth characteristics of BFU-Es.[9] Studies of hemoglobin biosynthesis performed on isolated colonies of CFU-E and BFU-E origin from normal bone marrow methyl cellulose cultures show virtually no synthesis of Hb F in CFU-E colonies, whereas Hb F is produced in colonies originated from BFU-Es.[14] Such data have been interpreted as indicating that the BFU-E-type progenitors, in contrast to more differentiated precursors (CFU-E), retain the ability to express Hb F synthesis.[9] The data on Hb F in the endogenous PV colonies are of interest in this context. This population of precursors appears to have different cycling characteristics than do normal peripheral blood BFU-Es, and it also appears to respond markedly to the addition of low doses of erythropoietin. If the expression of Hb F reflects, as we have suggested,[9] differences in degree of differentiation of progenitor cells giving rise to erythroid clones, the precursors of the endogenous colonies should be less differentiated than normal bone marrow CFU-E. In this context it is of interest that the PV patients we studied had normal or near-normal in vivo levels of Hb F (Table 1). The absence of elevated Hb F synthesis in vivo may indicate that the progenitors of the endogenous colonies undergo further differentiation in the bone marrow microenvironment.

The reasons for the decrease in Hb F production in the cultures of 3 of the 4 patients after erythropoietin was added to the PV cultures remain unknown. Studies of G6PD AB heterozygous patients suggest that the normal (i.e., non-PV) lines of progenitor cells that are suppressed in vivo give rise to erythroid colonies in culture when higher concentrations of erythropoietin are used.[3] It is thus possible that the decrease in Hb F when higher levels of erythropoietin are used reflects an increasing participation of non-PV BFU-Es that happen to direct lower levels of Hb F synthesis than do the PV precursors. It is also possible that the decrease in Hb F production reflects modulatory effects of erythropoietin (or contaminants of the erythropoietin preparations) on Hb F production in erythroid bursts. These and other possibilities are currently under investigation.

The patients whose cells were used in this study were selected to have normal or near-normal levels of Hb F in vivo. Although the majority of patients with PV produce normal levels of Hb F, in a few patients striking elevations of F cells are observed.[15] The reasons for the in vivo variation in Hb F production in this clonal disorder remain unclear. Cultures of PV precursors may provide an approach for investigating the mechanisms of the in vivo heterogeneity of Hb F production in polycythemia vera.

ACKNOWLEDGMENT

A portion of this work was conducted through the Clinical Research Center facility of the University of Washington supported by the National Institutes of Health (Grant RR-37). The human urinary erythropoietin was partially purified by Dr. P. Dukes (Hematology Research Laboratory, Children's Hospital of Los Angeles) and was generously supplied by the Blood Resources Branch of the National Heart, Lung ahd Blood Institute.

REFERENCES

1. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L: Polycythemia vera: Stem-cell and probable clonal origin of the disease. N Engl J Med 295:913, 1976

2. Prchal JF, Axelrad AA: Bone marrow response in polycythemia vera. N Engl J Med 289:1382, 1974

3. Prchal JF, Adamson JW, Murphy S, Steinmann L, Fialkow P: Polycythemia vera. The in vitro response of normal and abnormal stem cell lines to erythropoietin. J Clin Invest 61:1044, 1978

4. Weatherall DJ, Clegg JB, Wood WG: A model for the persistence or reactivation of fetal haemoglobin production. Lancet 2:660, 1976

5. Wasserman LR: The management of polycythemia vera. Br J Haematol 21:371, 1971

6. Wood WG, Stamatoyannopoulos G, Lim G, Nute PE: F-cells in the adult: Normal values and levels in individuals with hereditary and acquired elevations of Hb F. Blood 46:671, 1975

7. Papayannopoulou Th, Nakamoto B, Buckley J, Kurachi S, Nute PE, Stamatoyannopoulos G: Erythroid progenitors circulating in the blood of adult individuals produce fetal hemoglobin in culture. Science 199:1349, 1978

8. Papayannopoulou Th, Nute PE, Kurachi S, Stamatoyannopoulos G: Consistent activation of fetal hemoglobin synthesis in cultured adult bone marrow cells. Blood 51:671, 1978

9. Papayannopoulou Th, Brice M, Stamatoyannopoulos G: Hemoglobin F synthesis in vitro: Evidence for control at the level of primitive erythroid stem cells. Proc Natl Acad Sci USA 74:2923, 1977

10. Lingrel JB, Borsook H: A comparison of amino acid incorporation into the hemoglobin and ribosomes of marrow erythroid cells and circulating reticulocytes of severly anemic rabbits. Biochemistry 2:309, 1963

11. Boyer SH, Belding TK, Margolet L, Noyes AN, Burke PJ, Bell WR: Variations in the frequency of fetal hemoglobin-bearing erythrocytes (F-cells) in well adults, pregnant women, and adult leukemics. Johns Hopkins Med J 137:105, 1975

12. Papayannopoulou Th, Bunn HF, Stamatoyannopoulos G: Cellular distribution of hemoglobin F in a clonal hemopoietic stem-cell disorder. N Engl J Med 298:72, 1978

13. Papayannopoulou Th, Rosse W, Stamatoyannopoulos G: Fetal hemoglobin in paroxysmal nocturnal hemoglobinuria. Evidence for derivation of the Hb F containing erythrocytes (F-cells) from the PNH clone as well as from normal hemopoietic stem cell lines. Blood 52:740, 1978

14. Kidoguchi K, Ogawa M, Karam JD, Martin AG: Preferential synthesis of fetal hemoglobin (Hb F) in culture by human erythropoietic precursors in the marrow and peripheral blood. Clin Res 26:350A, 1978

15. Hoffman R, Papayannopoulou Th, Landaw S, Wasserman LR, DeMarsh QB, Stamatoyannopoulos G: Fetal hemoglobin in polycythemia vera. Cellular distribution in fifty unselected patients. Blood (submitted)

1979 53: 446-454

Hb F production of endogenous colonies of polycythemia vera

T Papayannopoulou, J Buckley, B Nakamoto, S Kurachi, PE Nute and G Stamatoyannopoulos

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.