Synthesis of Hemoglobin F in Adult Simian Erythroid Progenitor-derived Colonies

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ABSTRACT The simian hematopoietic system is known to respond to anemic stress with the production of erythrocytes containing large amounts of fetal hemoglobin. To determine the regulatory mechanism responsible for hemoglobin F (HbF) production in stress erythropoiesis, adult simian bone marrow cells were cultured in plasma clots in the presence or absence of erythropoietin and burst-promoting activities, and the HbF content of progenitor-derived colonies was determined by radioimmunoligand assay. Three classes of erythroid progenitors were detected: BFU-E, CFU-E, and a very mature cohort of dense highly erythropoietin-responsive cells (HERC). These classes varied in inverse proportion to their maturity with respect to their potential for HbF accumulation in the colonies to which they give rise. Both erythropoietin and burst-promoting activity stimulated HbF production, particularly in colonies derived from immature progenitors. For example, under conditions of high erythropoietin stimulation, BFU-E colonies contained 13.7–37.7% HbF, CFU-E colonies contained 2.8–13.5% HbF, and HERC colonies 0–1% HbF.

These results suggest that under nonstress conditions simian erythrocytes are derived almost entirely from HERC progenitors and proerythroblasts in which gamma chain synthesis is suppressed. During stress erythropoiesis, augmented HbF accumulation could be explained by the rapid entrance into the marrow of proerythroblasts directly derived from immature progenitors. Gamma chain production in these proerythroblasts is additionally regulated by the changes in environmental erythropoietin and burst-promoting activities.

INTRODUCTION

The regulation of fetal hemoglobin (HbF)[1] production in adult erythrocytes has been the subject of intensive investigation (1). On the basis of erythroid cell culture experiments and qualitative fluorescent antibody analysis, Papayannopoulou et al. (2) originally suggested that the extent of reactivation of HbF synthesis in cultured erythroid colonies depends on the state of maturity of the erythroid progenitors from which the colonies are derived. Recently, other investigators have found evidence that the reactivation of HbF production in adult erythroid colonies may be less contingent on the state of progenitor maturity than on the particular environmental conditions under which the progenitor was cultured. For example, Peschle et al. (3) presented evidence that erythroid burst-forming unit (BFU)-E subcolonies and colony-forming unit (CFU)-E colonies synthesize equal relative amounts of HbF, and Dover et al. (4) have shown that the percentage of HbF-laden cells in colonies derived from adult human bone marrow BFU-E does not differ significantly from that found in the more mature CFU-E colonies. Furthermore, Terasawa et al. (5) have found that factors derived from T cells, such as burst-promoting activity (BPA), may influence HbF production in erythroid colonies. This controversy has been further complicated by the finding that gamma and beta globin

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[1] *Abbreviations used in this paper*: BFU, burst-forming units; BPA, burst-promoting activity; CFU, colony-forming units; HbA, adult hemoglobin; HbF, fetal hemoglobin; HERC, highly erythropoietin-responsive cells.

chains are expressed asynchronously in culture (6–8), which leaves open the possibility that experimental results based on radiolabeled biosynthetic studies might be strongly influenced by the extent of colony hemoglobinization at the time the cultures are assayed (9).

DeSimone et al. (10) have shown that hypoxic or phenylhydrazine-stressed baboons are capable of very brisk erythropoiesis, during which peripheral blood levels of HbF rise from <1 to ≥40% of the total erythrocyte hemoglobin, and that HbF is the predominant hemoglobin synthesized in the cultured erythroid progenitor-derived colonies of such experimental animals (11). These findings, together with our previous observations that the erythroid colonies of normal rhesus monkeys appear rapidly in plasma clot culture, lyse soon after maximal hemoglobinization, and incorporate relatively large amounts of labeled leucine into gamma chains (12), prompted our decision to study these animals in more detail. Our purpose was to define the interrelated influences of erythroid progenitor maturity and of two hormonal factors, erythropoietin and BPA (both known to increase in circulating activity during stress erythropoiesis) (13, 14) on globin gene expression in vitro. In order that our conclusions not be influenced by potential artifacts of short-term labeling, we used instead a radioligand immunoassay to measure quantitatively the amounts and proportions of HbA and HbF found in the pooled completed simian bone marrow erythroid colonies cultured in plasma clots. Our results show that the potential for HbF expression in colonies derived from erythroid progenitors is indeed inversely proportional to progenitor maturity, but that culture conditions determine the actual extent of HbF production in the colonies derived from immature progenitors that are capable of significant gamma gene expression.

METHODS

Bone marrow aspiration and mononuclear cell preparation. Seven normal adult rhesus monkeys (*Macaca mulatta*) were the subjects of 13 unique experiments. The animals were anesthetized with ketamine (100–200 mg injected i.m.) and bone marrow samples aspirated from the posterior iliac crests. Multiple aspirates were diluted 1:1 with alpha medium (15) and centrifuged on 15 ml Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) for 30 min at 400 *g* and 22°C to separate the nucleated hematopoietic cells (interface layer) from mature erythrocytes (pellet). The interface was collected, resuspended in 10 ml alpha medium, and recentrifuged under the same conditions on 5 ml Ficoll-Hypaque for 30 min to eliminate most of the remaining erythrocytes. The interface was again collected and an aliquot fixed and stained to determine the percentages of mature erythrocytes and recognizable immature erythrocytes found in the final cell suspension. Cell suspensions prepared in this way generally had <4% erythrocytes and essentially no recognizable erythroid precursors. The cells were then washed three times in alpha medium, counted, and resuspended in alpha medium at 10 times the final plasma clot culture concentration.

Cell culture. To obtain sufficiently large numbers of erythroid colonies, mononuclear cells were plated at 5–7 × 10^5 cells/ml, using the plasma clot system described elsewhere (15, 16). Anemic sheep plasma erythropoietin (Connaught Medical Research Laboratories, Willowdale, Ontario, Canada, Step III) was reconstituted with National Collection of Type Cultures (NCTC)-109 (15) and added to the culture mixture. In certain experiments Mo-line hairy cell leukemia supernate (17), generously provided by Dr. David Golde, was added as a source of BPA to the plasma clot mixture (10% by volume, replacing an equal volume of NCTC-109). Three to six 0.1-ml vol clots were used for morphologic studies and colony counts, and 30–50 0.1-ml clots were pooled for each data point in the radioligand immunoassay of adult hemoglobin (HbA) and HbF. In those cases where exact quantitation of input background hemoglobin was desired, 50 0.1-ml cultures were prepared with mononuclear cells that had been irradiated with 1,500 rad at 500 rad/min before plating. Initial studies showed no erythrocyte lysis at this level of radiation, although all cell proliferation was prevented and no hemoglobinized colonies formed in these clots. The irradiated clots were incubated, harvested, and assayed together with experimental samples to provide an estimate of the background hemoglobin in the cultures derived from mature erythrocytes added at the time of culture initiation.

Simian CFU-E colonies were harvested on day 3 in culture, whereas simian BFU-E colonies were harvested on day 7–9 (see text). On the day of harvest, several 0.1-ml clots were squashed, fixed, and benzidine stained (15, 16). Clots for hemoglobin analysis were pooled, gently suspended in 15 ml of alpha medium and washed twice at 300 *g* for 10 min at 4°C. 10 μl of packed sheep erythrocytes were added to provide carrier hemoglobin, and the mixture was once again centrifuged at 300 *g* for 10 min at 4°C. The supernate was removed and discarded. The pelleted clots and carrier erythrocytes were resuspended in 200 μl of sterile distilled water. These mixtures were sonicated (3–4 5-s bursts, at low output) and the resulting cell lysates centrifuged at 800 *g* for 10 min. The hemoglobin-containing supernate was removed and the volume measured. These sonicates were maintained at 4°C until they were assayed.

The extent of erythroid precursor maturity and the level of hemoglobinization within the precursors found in pooled colonies was determined by releasing hemoglobinized cells from plasma clots with gentle pronase treatment (15). The released cells were then washed, centrifuged, and stained with Wright-Giemsa stain.

Radioimmunoligand assay. The hemoglobin contained in the plasma clot sonicates was assayed using a radioligand assay for HbA and HbF as described in detail elsewhere (18). Briefly, several dilutions of each sample were incubated for 0.5 h with an excess of each of two antisera, one specific for fetal Rhesus hemoglobin alone, the other equally reactive with HbF or HbA. The immune complexes thus formed were bound to a protein A-bearing strain (Cowan 1) of *Staphylococcus aureus*. The immunologically unreactive hemoglobins were removed by washing the pelletted bacteria. The hemoglobin specifically retained was then quantitated by its ability to bind a calibrated sample of ^{125}I-haptoglobin. Standard curves were constructed under identical conditions using known amounts of Rhesus HbF and HbA and were included in each assay.

Initial calibration experiments were performed to determine the sensitivity of the assay. Dilutions of adult and fetal erythrocytes in three different proportions ranging from 5 to 50% HbF were made and aliquots of 10^5–10^6 erythrocytes were placed in plasma clots, allowed to stand for 1 h, and harvested, washed, and sonicated as outlined above. The sonicates were assayed in a blind determination and the results of the radioligand immunoassay compared to the known inputs of these hemoglobins. The fraction of HbF determined by the assay was within 5% of the expected values.

Density centrifugation. Estimates of progenitor cell density were made by analysis of Percoll density centrifugation behavior as described by Salvado and Sytkowski (19). Briefly, 9 ml of Percoll were made isotonic by the addition of 0.72 ml of NCTC-109 and 0.28 ml of sterile H_2O. The pH was adjusted to 7.4 with 1.0 N HCl. This stock solution was termed 100% Percoll. Lower density Percoll solutions were prepared by diluting this 100% Percoll solution with NCTC-109. The density of these dilutions were calculated from their refractive indices as described (19). Block gradients were constructed in 15-ml centrifuge tubes using four blocks of 80, 70, 60, and 50% Percoll (bottom to top) layered gently on top of each other. These four blocks were overlayed with 30–60 × 10^6 washed bone marrow cells suspended in 3 ml of a 1:1 mixture of 50% Percoll and NCTC-109 media. The gradients were centrifuged at room temperature, 400 *g*, for 40 min. Each Percoll block and each interface was collected, and the cells contained in each of these fractions were washed, counted, and cultured as described above.

RESULTS

Morphology of simian progenitor-derived colonies. Fig. 1A and C depicts simian CFU-E and BFU-E derived colonies cultured for 3 and 7 d, respectively, in the presence of 2 U/ml of erythropoietin. CFU-E colonies contain 8–16 hemoglobinized cells while BFU-E colonies contain 100–200 cells. Fig. 1B depicts the morphology of colonies that proliferate and hemoglobinize in 3 d in the absence of added erythropoietin (see below). They contain 4–8 hemoglobinized cells.

Fig. 2 shows a representative Wright-stained cytocentrifuged preparation of cells released by pronase treatment from plasma clots containing BFU-E colonies on the day of peak colony hemoglobinization. Relatively immature erythroid cells predominate. There was no significant difference between the mor-

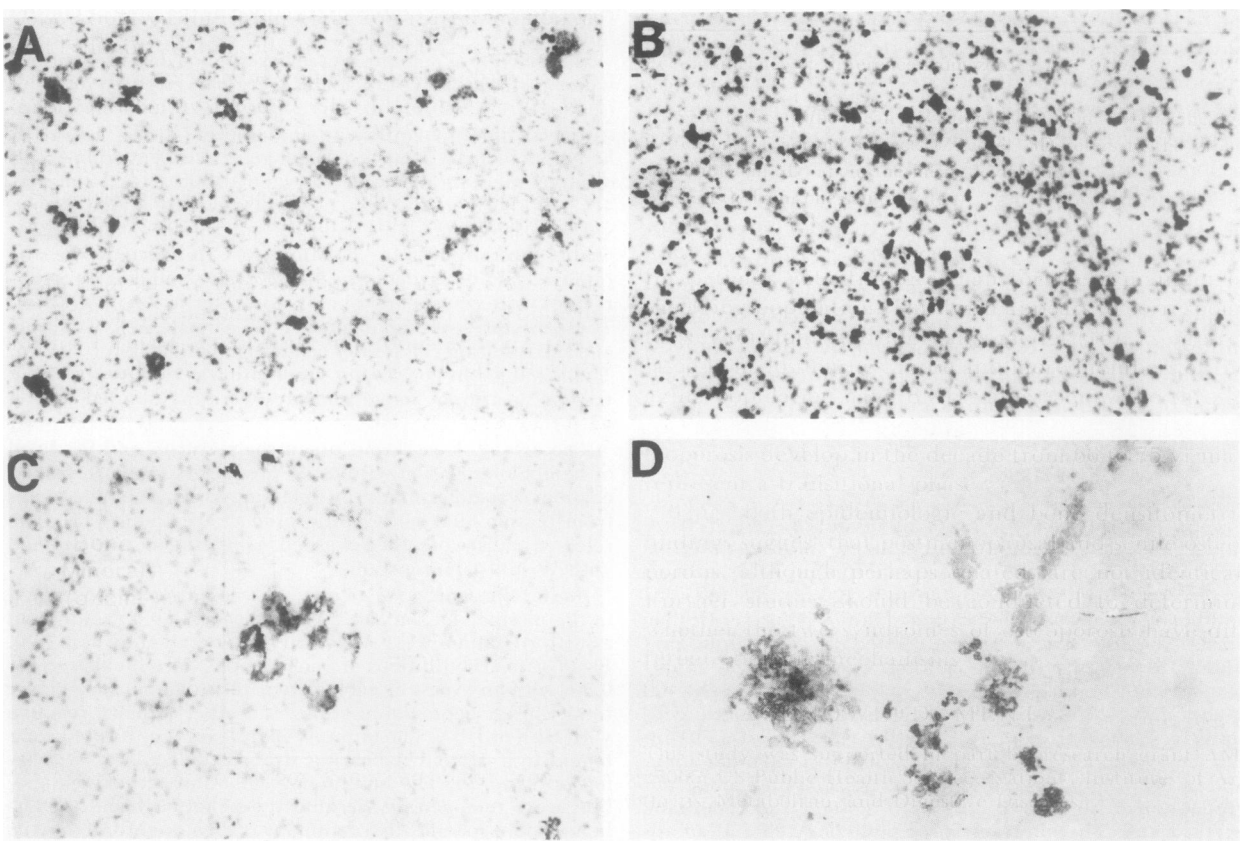

FIGURE 1 Photomicrographs at equal magnification of hematoxylin and benzidine stained simian CFU-E (A), HERC (B), 7-d BFU-E (C), and 9-d BFU-E-derived colonies (D). The HERC-derived colonies were grown in the absence of added erythropoietin.

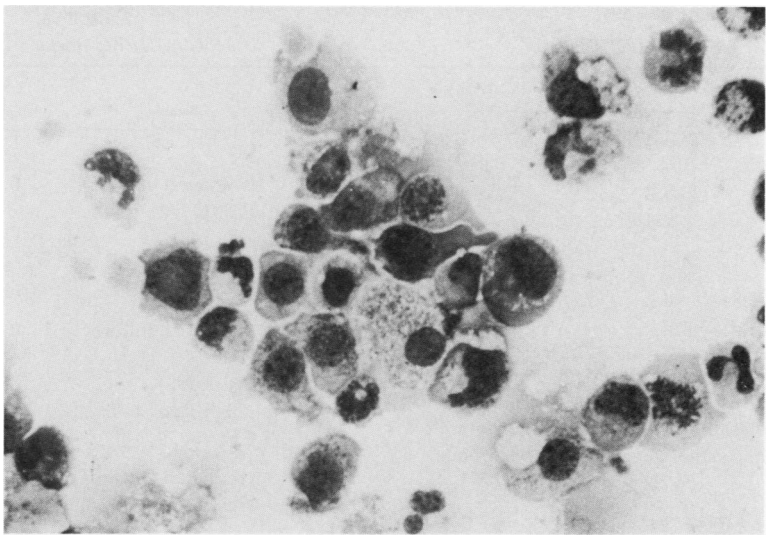

FIGURE 2 Photomicrograph of a typical cluster of erythroid cells released by pronase treatment from a plasma clot culture of simian BFU-E-derived colonies, cytocentrifuged and stained with the Wright-Giemsa technique. Most of the erythroid cells are midnormoblasts, but some basophilic erythroblasts are present.

phologically perceived level of erythroblast maturity in cells released from CFU-E-derived colonies and those released from BFU-E-derived colonies. Although some selective losses of mature cells may occur during pronase treatment, these results show that differences in HbF content (see below) between CFU-E- and BFU-E-derived colonies cannot be readily ascribed to putative differences in the extent of colony maturation.

Effects of increasing activities of crude erythropoietin on simian colony frequency and morphology. Previous studies have demonstrated that the process of erythroid progenitor maturation involves a gradual decrease in the level of erythropoietin required for maximal colony growth (20). Fig. 3 is a representative erythropoietin dose-response curve showing the effect of increasing activities of crude erythropoietin on simian CFU-E- and BFU-E-derived colony numbers. As expected, peak CFU-E colony frequency occurs at a lower level of erythropoietin than does peak BFU-E colony frequency. Although there is no BFU-E colony formation in the absence of added erythropoietin, ~20% of maximal CFU-E colony growth is expressed at zero erythropoietin, where zero represents no addition of sheep plasma erythropoietin to the culture. This subset of CFU-E, which proliferates in the absence of added erythropoietin (Fig. 1B), can be increased to include ~40% of maximal CFU-E frequency if the marrow cells are initially incubated for 1 h in 2 U/ml of erythropoietin, and then washed twice before the start of the culture. Colonies formed from these cells hemoglobinize slightly earlier than those derived from CFU-E. We refer to these CFU as highly erythropoietin-responsive cells (HERC). Table I provides a summary of differences between CFU-E and HERC and the colonies to which they give rise.

Determination of amounts and proportions of HbA and HbF in simian BFU-E-, CFU-E-, and HERC-derived colonies. Table II summarizes the results of eight experiments in which simian erythroid progenitor-derived colonies were cultured at 2 U of erythropoietin/ml, as described above. The resultant CFU-E- and BFU-E-derived colonies were harvested, sonicated, and the released hemoglobin assayed with the radioligand immunoassay. The data indicate that total

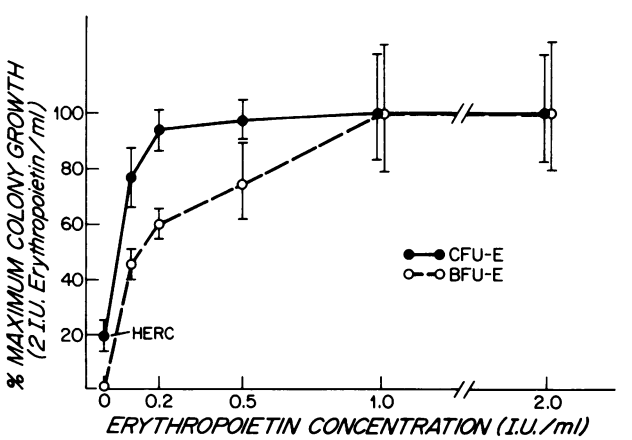

FIGURE 3 Typical erythropoietin dose-response curve for adult simian CFU-E- and BFU-E-derived colonies. The CFU-E detected at zero added erythropoietin are designated as HERC-derived colonies.

TABLE I
Comparison of CFU-E and HERC

	CFU-E	HERC
Colonies/10^5	128–1,409	78–546
Days to maximal colony formation	3–4	2–3
Cells per colony	8–16	4–8
Erythropoietin added for maximal colony formation, *U/ml*	0.2	0
Peak on Percoll density gradient, g/cm^3	1.067–1.079	1.092–1.104

CFU-E were measured at 2 U erythropoietin/ml culture and HERC at either no added erythropoietin or after 1 h exposure to 2 U/ml erythropoietin and no further addition of the hormone.

hemoglobin recovery closely approximated the expected hemoglobin content of the colonies, if one assumes a mean hemoglobin content of 20 pg/cell and an average of 10 cells/CFU-E-derived colony and 200 cells/burst. Furthermore the HbF production of BFU-E-derived colonies exceeded that of CFU-E-derived colonies on a relative and absolute basis. In four experiments, set out in Table III, HERC colony growth was sufficiently greater than the background hemoglobin attributed to mature erythrocytes (estimated from parallel studies of irradiated culture plates; see Methods) to allow a reliable determination of their hemoglobin proportions. These HERC colonies were defined as the subset of CFU-E that develop in the absence of added erythropoietin. They contained virtually no HbF.

TABLE III
HbF in HERC-derived Colonies

Unique experiment no.	Sample	Colonies/10^5	Hemoglobin	HbF
			ng/ml	%
2	Irradiated culture	0	21	0
	HERC			
	0 Epo	197±40	238	0
	1 h Epo	377±41	324	0
	CFU-E	907±68	627	9.9
3	Irradiated culture	0	ND	—
	HERC			
	0 Epo	109	144	0
	1 h Epo	78±28	138	0
	CFU-E	1,130±112	1,595	13.5
5	Irradiated culture	0	188	0
	HERC			
	0 Epo	202±27	406	0
	1 h Epo	536±82	349	0
	CFU-E	1,153±87	847	2.8
9	Irradiated culture	0	78	0
	HERC			
	0 Epo	ND		
	1 h Epo	546±109	519	0.2
	CFU-E	980±218	1,925	1.0

Cultures performed for the times and under conditions described in text. Irradiated cultures were done to determine nanograms per milliliter of hemoglobin carried inadvertantly into the culture as mature erythrocytes. The assay of hemoglobin was not performed in the irradiated culture of experiment 3, but inspection of the cultures on day 3 revealed that erythrocytes were virtually absent. HERC were cultured at no added erythropoietin (0 epo) or after 1 h exposure to 2 U erythrpoietin/ml (1 h epo).

TABLE II
HbF in CFU-E- and BFU-E-derived Colonies

Unique experiment no.	CFU-E	Hemoglobin	HbF	BFU-E	Hemoglobin	HbF
	colonies/10^5	*ng/ml*	%	*colonies/10^5*	*ng/ml*	%
1	1,327±6	1,235	8.2	43±5	929	37.7
2	907±68	627	9.9	39±4	563	33.5
3	1,130±112	1,595	13.5	52±5	2,996	25.4
4	1,199±98	1,054	7.6	85±10	1,333	34.8
5	1,153±87	847	2.8	66±11	963	31.6
6	128±25	276	13.2	35±7	996	27.2
7	727±42	1,148	12.1	41±9	936	18.9
8	1,409±100	2,207	3.9	123±13	2,568	13.7

In this and all subsequent tables, except where indicated, CFU-E and BFU-E cultures were performed at 2 U erythropoietin and 5×10^5 marrow cells/ml of plasma clot. Therefore, colonies/10^5 must be multiplied by 5 in order to compare colony number with total recovered hemoglobin. The latter and percentage of HbF were extracted from 3- to 5-ml clot, measured at several dilutions and quantified from a standard curve as described in the text. Since the assay is linear, the dilutions gave virtually identical results and therefore a single value for both total hemoglobin and HbF is provided.

TABLE IV
*Effect of Erythropoietin and Culture Time on HbF Production in BFU-E-derived Colonies (10)**

Time in culture	0.5 U erythrpoietin/ml			2.0 U erythrpoietin/ml		
	Colonies/10^5	Hemoglobin	HbF	Colonies/10^5	Hemoglobin	HbF
d		*ng/ml*	%		*ng/ml*	%
7	19±4	1,063	17.1	16±7	854	24.0
8	23±2	1,217	22.7	9±3	845	49.7
9	14±8	886	38.3	9±2	771	54.4

The decline in colony number between day 7 and 9 is not statistically significant. Note the absolute as well as relative increase in HbF between days 7 and 9.
* Unique experiment number.

Effects of culture time on the HbF content of BFU-E-derived colonies. Because other investigators have found evidence that gamma gene expression declines with length of time in culture, (8, 21) it was important to investigate the effect of culture time on HbF content in BFU-E-derived colonies. Table IV shows the BFU-E colony frequency and the fraction of HbF produced by BFU-E-derived colonies cultured at 0.5 or 2 U/ml erythropoietin and harvested on days 7, 8, and 9 of culture. At both levels of erythropoietin activity, colony counts were maximal at days 7–8 and declined thereafter as colony lysis occurred. In general, colonies that hemoglobinized on day 7 contained fewer and smaller subcolonies than those detected on day 9 (Fig. 1C, D). Despite the decline in colony frequency the relative and absolute proportion of HbF in the pooled colonies continued to rise from day 7 through day 9. Furthermore, the proportion of HbF was regularly higher in colonies cultured in the presence of 2 U of erythropoietin than in those cultured in the presence of 0.5 U of erythropoietin/ml.

Effects of increasing activities of crude erythropoietin on the proportion of HbF in erythroid colonies. Table V shows the effects of increasing activities of erythropoietin on colony frequency and the proportion of HbF in CFU-E-derived colonies. Note that, as described above, the colonies which hemoglobinized in the absence of added erythropoietin contained <1% HbF. Between 0 and 0.5 U/ml of erythropoietin, there was a gradual increase in colony frequency and percentage of HbF, whereas from 0.5 to 2 U/ml erythropoietin there was almost a twofold increase in the proportion of HbF without a significant increase in either colony frequency or recovered hemoglobin.

Table V also presents the effects of increasing erythropoietin activity on HbF accumulation and colony frequency in BFU-E-derived colonies. The proportion of HbF generally rose by a factor of 10 as erythropoietin activity was increased from 0.1 to 2 U/ml. As was observed with CFU-E-derived colonies the increase in the proportion of HbF detected between 0.5 and 2 U/ml of erythropoietin was not accompanied by a corresponding increase in colony frequency. It is important to emphasize that the proportion of HbF in BFU-E colonies cultured at the lowest erythropoietin concentrations utilized (0.1 U/ml) was much lower than the proportion observed in CFU-E-derived colonies cultured at the highest erythropoietin concentration utilized (2.0 U/ml; Discussion).

Table VI provides the results of five separate studies of BFU-E-derived colony frequency and hemoglobin formation and confirms that the relative proportion and absolute content of HbF in such colonies increased between 0.5 and 2 U of erythropoietin irrespective of colony frequency or total hemoglobin recovery.

TABLE V
Influence of Crude Erythropoietin on Erythroid Progenitor-derived Colonies

	Erythropoietin activity (*U/ml*)			
	0	0.1	0.5	2.0
CFU-E (7)*				
Colonies/10^5	223±20	316±21	687±32	727±20
Hemoglobin, *ng/ml*	305	1,331	1,400	1,148
HbF, %	0.6	1.3	6.9	12.1
BFU-E (5)*				
Colonies/10^5	0	38±3	61±2	66±11
Hemoglobin, *ng/ml*	13	858	1,637	962
HbF, %	‡	3.6	14.2	31.6

The increase in HbF as a function of erythropoietin activity is absolute as well as relative and has no predictable relationship to the calculated hemoglobin per colony.
* Unique experiment number.
‡ Value below limits of accurate detection.

TABLE VI

Influence of Crude Erythropoietin on HbF Accumulation in BFU-E-derived Colonies

Unique experiment no.	Erythropoietin activity	Colonies/10^5	Hb	HbF
	U/ml		*ng/ml*	*%*
4	0.5	60±5	1,502	17.1
	2.0	85±10	1,333	34.8
5	0.5	61±2	1,637	14.2
	2.0	66±11	963	31.6
8	0.5	148±28	3,227	7.2
	2.0	123±13	2,568	13.7
11	0.5	26±5	110	18.9
	2.0	59±10	120	25.5
12	0.5	19±4	1,063	17.1
	2.0	16±7	854	24.0

The increase in HbF as a function of erythropoietin activity is both absolute and relative and has no predictable relationship to the calculated hemoglobin per colony.

TABLE VII

Effect of BPA on Colony Formation and HbF Expression at 2 U Erythropoietin/ml

Unique experiment no.	Colony type	BPA* added	Colonies/10^5	Hemoglobin	HbF
				ng/ml	*%*
2	BFU-E	−	39±4	563	33.5
		+	60±14	741	46.5
7	CFU-E	−	727±42	1,148	12.1
		+	742±85	1,220	10.1
	BFU-E	−	41±9	936	18.9
		+	29±8	808	21.0
8	CFU-E	−	1,409±100	2,207	3.9
		+	1,564±102	2,424	2.8
	BFU-E	−	123±13	2,568	13.7
		+	145±10	1,968	32.1
11	BFU-E	−	59±10	120	25.5
		+	60±7	252	61.0
13	CFU-E	−	128±25	276	13.2
		+	134±38	233	15.5
	BFU-E	−	70±14	996	27.2
		+	24±4	575	35.7

* The supernate of a malignant T-cell line was added as a source of BPA to the plasma clot mixture (10% by vol) replacing an equal volume of NCTC-109. BPA induced a relative and absolute increase in HbF in the BFU-E-derived colonies developed in experiments 2, 8, and 11, only a relative increase in experiment 13, and had no effect in experiment 7. There is no predictable relationship of HbF content to calculated hemoglobin per colony.

Effects of BPA on simian erythroid colony frequency and proportion of HbF. Table VII shows the effects of the addition of T-cell-derived BPA to CFU-E and BFU-E colonies cultured in the presence of 2 U/ml of erythropoietin. BPA had no effect on CFU-E colony frequency or HbF content, whereas in four of five experiments BPA slightly to markedly increased the proportion of HbF contained in the BFU-E-derived colonies without any predictable relationship to colony frequency or hemoglobin recovery.

DISCUSSION

This study was designed to investigate the effects of progenitor maturity and culture conditions on simian HbF production in culture. The investigation used techniques by which the relative and absolute HbF content of progenitor-derived colonies could be reliably assayed. The results show that both maturation and environmental conditions play separate roles in the regulation of HbF production. The finding that, under identical culture conditions, BFU-E consistently generate colonies with 2–4 times the proportion of HbF contained by the more mature CFU-E-derived colonies shows that the potential for HbF production is in general inversely proportional to the maturity of the progenitor cell from which the colony was derived. Because the peripheral blood of the unstressed rhesus monkey contains <1% HbF, this model predicts the existence of a cohort of very mature erythroid progenitors which would give rise to colonies with little or no HbF. The characteristics of this group of progenitors should include exquisite erythropoietin sensitivity (and hence responsiveness to ambient levels of erythropoietin), small colony size, and early hemoglobinization. The growth characteristics of such CFU have been previously described in murine and ovine systems (22–25). The HERC, which produce 0–1% HbF, fulfill all of these requirements. Since we could not detect proerythroblasts in the interface layer after initial Ficoll-Hypaque preparation, we do not believe that HERC are identical to proerythroblasts. Further attempts at HERC concentration using physical separation techniques are now in progress. At present, we conclude that HERC represent a subset of relatively dense, very mature, CFU, at a stage of maturation somewhere between CFU-E and proerythroblasts. They produce colonies of erythroid cells containing almost 100% HbA.

On the basis of this support for the progenitor-maturity-based model of HbF regulation, it would be tempting to conclude that the increased HbF production observed in association with increments of crude erythropoietin activity in culture is attributable to recruitment of increasingly immature progenitors with higher erythropoietin requirements and progressively

greater potentials for HbF production. Certainly, this phenomenon accounts for part of the erythropoietin dose-response effect on HbF in the CFU-E cohort. In such experiments the essentially 100% HbA contributed by HERC colonies with no added erythropoietin is progressively diluted by hemoglobin contributed by the more immature CFU-E colonies containing significant amounts of HbF. However, the finding that, at levels of erythropoietin >0.5 U/ml, there continues to be a sharp rise in the HbF percentage without a corresponding rise in colony numbers suggests that, independent of recruitment effects, some activities present in crude erythropoietin are capable of directly stimulating HbF synthesis. Other investigators have attributed this crude erythropoietin dose-response effect to BPA contaminating the crude erythropoietin (5).

Additional evidence for direct stimulating effects of crude erythropoietin on HbF synthesis can be found in an analysis of HbF accumulation in BFU-E-derived colonies at low levels of erythropoietin. At 0.1 U/ml of erythropoietin, BFU-E colonies accumulated only 3.6% HbF. If the erythropoietin dose-response effect were due entirely to recruitment phenomena, the most mature BFU-E should never produce less HbF than the most immature CFU-E. Because CFU-E colonies had almost entirely lysed by the time BFU-E colonies were harvested and because BFU-E colonies had not begun to hemoglobinize on day 3, when CFU-E colonies were harvested, these results cannot be due to contamination of CFU-E-derived colonies by BFU-E-derived colonies or vice versa. Rather, these results analyzed together are most compatible with a model of HbF regulation involving two factors: First, the potential for HbF synthesis in vitro seems to be determined by the maturity of the progenitor from which the colony arose. Thus, BFU-E have an inherently greater potential for HbF synthesis than do CFU-E, which in turn have a greater potential for HbF synthesis than do HERC. Second, the actual amount of HbF synthesis in colonies derived from immature progenitors capable of substantial HbF synthesis is determined by the culture conditions. Thus, although BFU-E-derived colonies are inherently capable of greater HbF synthesis than CFU-E-derived colonies, at low activities of crude erythropoietin this potential capacity is not fully realized. In essence, the level of maturity of a simian progenitor determines the upper limit of HbF synthesis in culture, but the culture conditions will determine the actual extent of HbF synthesis up to that limit.

Other investigators have shown that at least in man, asynchrony in the expression of gamma and beta chains production results in a decline in overall HbF synthesis and accumulation as time in culture increases (21). In contrast, we found that the relative proportion and absolute content of HbF in pooled BFU-E colonies increased as time in culture is protracted. The differences in synthetic ratios would be reconciled in part if we assume that HbF-rich colonies tend to persist in culture while the HbA-rich colonies (derived from more mature progenitors) begin to lyse. Thus, even if relative gamma chain synthesis declines in each individual colony as hemoglobinization proceeds (i.e., the gamma/beta + gamma synthetic ratio decreases), the relative accumulation of gamma globin in the residual colonies which have not yet lysed would increase owing to preferential loss of HbA-rich colonies initially derived from more mature progenitors. On the other hand, relative lysis of mature colonies cannot totally explain the phenomenon because we found that the absolute amount of HbF in the culture also increases with time. Since environmental conditions have a profound effect on HbF accumulation in BFU-E-derived colonies, the observed differences between laboratories are probably the results of different culture techniques.

The effects of BPA on HbF accumulation are not established. Previous studies have suggested that the influence of BPA on hemoglobin accumulation is primarily directed at colonies derived from immature progenitors (5, 26). Our results showing that BPA has little or no effect on the hemoglobin accumulation of relatively mature simian CFU-E-derived colonies are in close agreement with such conclusions. Although our experiments in which BPA activity was added to BFU-E did not provide absolutely consistent results, in most of the studies the addition of this product of a malignant T-cell line did result in an increase in HbF accumulation of modest-to-striking proportions in BFU-E-derived colonies. This result suggests that in conditions of stress, when both erythropoietin and BPA are increased, the two hormones may collaborate in some fashion to induce maximal HbF production in the colonies derived from immature progenitors.

The implication of these in vitro studies for the regulation of in vivo erythropoiesis can be best appreciated in the context of the experiments of Alpen and Cranmore, (27, 28) who examined cellular dynamics and the regulation of erythropoiesis in the normal and acutely bled dog and those of Deubelbeiss et al. (29), who measured the total proerythroblast content of canine marrow. The former investigators labeled hemoglobinizing proerythroblasts in vivo with ^{59}Fe and sequentially analyzed the proportion of labeled and unlabeled proerythroblasts to measure their renewal from an unlabeled pool and their doubling times. In the bled animals there was a rapid surge of unlabeled cells into the labeled proerythroblast compartment without any change in the doubling time of the latter or in the number of divisions between proerythroblasts and reticulocytes. Alpen and Cranmore therefore con-

cluded that during stress erythropoiesis there was "an influx of a larger proportion of freshly differentiated proerythroblasts into the hemoglobinizing cohort" (28). Further analysis of their data and that of Dubelbeiss et al. (29) show that under normal conditions self-replicating proerythroblasts largely maintain the erythrocyte pool, being renewed from the immature progenitor compartment at a rate of <10%/d.[2] In contrast, in the bled animal, nearly the entire burden of accelerated reticulocyte production is borne by rapid influx from the immature progenitor compartment into an expanded proerythroblast pool, a renewal that approaches a rate of 120%/d.

These data and our own suggest that the striking increase in HbF-laden cells that accompanies simian stress erythropoiesis is attributable at least in part to the entry into the simian marrow of proerythroblasts derived from immature progenitors that are forced prematurely into terminal differentiation. We propose that unlike erythroblasts derived largely from the HERC and self-renewing proerythroblast compartments, the gamma globin genes of which are suppressed, the erythroblasts proximally derived from immature progenitors (CFU-E and BFU-E) are recruited into differentiation before suppression of gamma genes is completed. In addition, hemoglobinization occurs in such proerythroblasts in the presence of the high levels of erythropoietin and BPA that are observed in many anemic states. Both appear to have the capacity to induce maximal amounts of gamma globin synthesis in erythroblasts in which gamma gene suppression is sufficiently incomplete. This combination of kinetic and environmental forces influences the number of F cells and the extent of HbF accumulation per cell in stress erythropoiesis.

Dover et al. have recently reported results of their studies of F cell production in vivo (7, 30) and in vitro (4, 31). They have confirmed that in certain hemoglobinopathic states such as sickle cell anemia and thalassemia, there is an increase in the number of erythrocytes containing detectable HbF (F cells). The amount of HbF per F cell is often no different in these conditions than that observed in the rare circulating F cells in normal individuals, ~4–8 pg of HbF/cell. On the other hand, the production of cells containing larger amounts of HbF (8–15 pg of HbF/cell) is a regular feature of states such as posthemorrhagic anemia, bone marrow transplantation, and marrow failure. Using single cell assays, Dover et al. (4) have shown that while erythroblasts derived from human BFU-E-derived colonies contain more HbF per cell than cells derived from CFU-E-derived colonies, there is no significant difference in the fraction of erythroblasts containing detectable HbF in these colonies. Moreover, the number of circulating F cells produced by an individual seems to be genetically determined, whereas the amount of HbF per F cell is not under simple genetic control (30). Thus a third factor, a genetic predisposition to express gamma genes in proerythroblasts derived from any progenitor together with the influences of progenitor maturity and environment combine to influence the total production of HbF in the various forms of stress erythropoiesis. Differences in genetic factors are perhaps most strikingly shown in the simian system. Though in general the capacity to produce HbF in response to stress is much more impressive in simians than in man, DeSimone et al. (10) have noticed a marked variability among families of baboons to respond to stress in this fashion.

The application of these results to human erythropoiesis has yet to be determined. Though certain biosynthetic and fluorescent antibody studies have suggested that reactivation of HbF synthesis is readily detected in human marrow cultures (2, 6), we found that F synthesis was much lower in human marrow than in human blood progenitor cultures (15), and our preliminary quantitative studies of normal human marrow CFU-E- and BFU-E-derived colonies show that HbF accumulation is only 20% or less of that exhibited by rhesus monkey erythroid colonies. Further studies are now in progress with which we hope to clarify the interaction of environmental conditions, hormonal levels, cellular dynamics, and genetic predispositions in the regulation of HbF production in humans.

[2] This conclusion is based on the following data: Dubelbeiss (29) measured the total proerythroblast content of canine marrow and found an average value of 0.17×10^9 proerythroblasts/kg body wt, or (assuming a blood volume of 70 ml/kg) 0.24×10^4 cells/μ^3 blood. Alpen and Cranmore (27, 28) found that the daily renewal rate of normal canine proerythroblasts from the progenitor compartment is 30%, or 0.07×10^4 cells/μ^3 blood/d. They also found that the mean number of divisions between the proerythroblast and reticulocyte compartment is 2.3. Thus, each proerythroblast contributes an average of 5 reticulocytes. The reticulocyte production of the dog is 5×10^4 cells/μ^3 blood/d. Therefore, 10^4 proerythroblasts/μ^3 blood are committed to daily reticulocyte production. A further 20% is committed to early death for a total commitment to differentiation of 1.2×10^4 cells/μ^3 blood/d. Hence, the contribution of proerythroblasts proximally derived from the progenitor pool (0.07×10^4 cells/μ^3 blood/d) to daily reticulocyte production is ~6%/d.

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