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Research Article

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ABSTRACT To explore the etiology of congenital hypoplastic anemia (CHA) or the Diamond-Blackfan anemia, erythropoietin responsive committed erythroid precursors were enumerated by the plasma clot method. These included blood and marrow erythroid burst-forming units (BFU-E) and marrow erythroid colony-forming units (CFU-E). The peripheral blood nucleated cells of 11 patients and the marrow cells of seven of these patients were examined. Studies were repeated in several patients during relapse and after induction of remission. BFU-E were undetectable in the marrow and blood of all but one relapsed patient, and the numbers of marrow CFU-E were depressed in all relapsed patients. Blood BFU-E remained low in all of the patients in remission. No evidence was obtained for suppression of normal CFU-E or BFU-E by CHA lymphocytes. Erythropoietin dose-response curves performed in two patients revealed a 10-fold increase in erythropoietin requirement for marrow CFU-E colony growth. This marked unresponsiveness to erythropoietin was strikingly improved by steroid therapy in one patient. We suggest that CHA is the result of a qualitative and/or quantitative deficiency of BFU-E. If BFU-E are produced, they must be relatively unresponsive to erythropoietin. The abnormal BFU-E give rise to erythropoietin unresponsive CFU-E and, thence, to proerythroblasts that are, in turn, trapped in that early stage of development because of their poor erythropoietic response. Hence, red cell production is deficient. Steroids appear to improve the erythropoietin response of CHA erythroid precursors.

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INTRODUCTION

The in vitro ability to culture normal erythroid precursor cells from human bone marrow and peripheral blood provides insight into the pathology of congenital and acquired disorders of erythropoiesis. Previous investigations in animal model systems indicate that at least two classes of morphologically undifferentiated erythroid precursor cells may be identified by in vitro cultivation techniques (1–4). These precursor cells differ in size and proliferative capacity. It is presumed that the precursor cells of highest proliferative capacity (erythroid burst forming units or BFU-E)[1] give rise in vivo to cells of lower proliferative capacity (erythroid colony forming units or CFU-E) and that morphologically recognizable marrow erythroid precursors arise from CFU-E. Adaptation of these techniques to culture of human erythroid precursor cells has revealed that these two classes of morphologically undifferentiated precursor cells also can be identified in human marrow (5, 6) although only BFU-E can be demonstrated in human peripheral blood (7, 8). The present report describes the results of our studies with human erythroid precursor cells in congenital hypoplastic anemia (CHA). This disease is an uncommon childhood defect in erythroid marrow cell differentiation characterized by absolute reticulocytopenia and a paucity of bone marrow erythroid cells more mature than proerythroblasts (9). In most cases, marrow erythroid cellularity and remission of anemia can be achieved by treatment with low doses of glucocorticoids. Remission is loosely defined in the management of this disorder. Sufficient steroid therapy is given to maintain the hemoglobin above 8.5 g without transfusion requirement. In most

[1] *Abbreviations use in this paper*: BFU-E, erythroid burst-forming units; CFU-E, erythroid colony-forming units; CFU-S, colony-forming unit—spleen; CHA, congenital hypoplastic amenia.

such remissions, the blood remains abnormal in many respects, exhibiting macrocytosis, poikilocytosis, increased fetal hemoglobin, and increased red cell i antigen. The term "partial remission," therefore, might be preferable. The responsiveness of individual patients is variable. Some require much higher doses than others to maintain remissions. Those who require high doses are described as "partial responders."

The molecular bases for the failure of erythroid differentiation and the response to steroids are not understood. A previous study of the number of CFU-E in CHA marrow showed that CFU-E were moderately reduced in patients who were not on prednisone therapy (10) and other investigators have claimed that the disorder may be the result of autocytotoxic lymphocytes directed against marrow CFU-E (11).

In this study we compared the number or function of BFU-E and CFU-E in marrow and peripheral blood from normal individuals and patients with CHA in relapse and remission. We found a decided decrease in marrow CFU-E in relapsed patients. We also observed a virtual absence of functioning BFU-E in both marrow and peripheral blood of CHA patients in relapse as well as a quantitative deficiency of functioning blood BFU-E while patients were in remission. We could not find evidence to suggest a cytotoxic basis for this deficiency of erythroid precursor colony formation. In two relapsed cases, we demonstrated an extraordinarily decreased sensitivity of CHA CFU-E to erythropoietin. In one of these cases there was partial recovery of erythropoietin sensitivity while on steroid therapy. On the basis of these data, we suggest that, at least in certain patients with this disease, erythroid failure may be due to reduced erythropoietin responsiveness of erythroid precursor cells. This diminished reactivity can be ameliorated by steroid therapy.

METHODS

Human subjects and blood and marrow samples

Controls. The normal blood and marrow samples were obtained from adult volunteers with informed consent, from anesthetized patients undergoing repair of scoliosis, and from hematologically normal children 5–8 yr of age who underwent marrow and blood studies as part of routine follow-up for acute lymphoblastic leukemia in remission. None of these children had received chemotherapy for over 1 yr.

CHA. The relevant clinical characteristics of the 11 patients with CHA are presented in Table I. All but one of the patients in remission (hemoglobin above 8 g/100 ml) required low doses of corticosteroids for maintenance. The seven patients in relapse (hemoglobins 7.5 g/100 ml or less) were not on corticosteroid therapy at the time of marrow and blood culture. Two patients were only partially responsive (cases 3 and 4) and two were totally unresponsive (cases 5 and 6) to prednisone therapy. In five relapsed patients, proerythroblasts constituted from 2 to 10% of the total nucleated cells in the marrow and from 40 to 100% of the very reduced numbers of marrow erythroid precursors. More mature marrow erythroid cells were very deficient. Case 6 was exceptional because there were no recognizable erythroblasts of any type present in her marrow. Case 7 was also unusual in that she was one of the older patients and had been in steroid-independent remission for 15 yr when anemia recurred. The bone marrow aspirate revealed an increased percentage of erythroid precursors, but there was also a marked increase in the percentage of proerythroblasts. In the 2 wk after the initial marrow aspirate, reticulocytopenia became evident. Steroid therapy was instituted and produced a prompt remission. The remission marrow during steroid administration revealed a normal percentage of proerythroblasts.

Samples. Blood was procured from all patients studied, and marrow samples were obtained from seven of these patients (on two or three occasions from three of them). Bone marrows were obtained only when patients were in relapse and the marrow aspiration procedures were clinically indicated. In one older patient (case 7), the bone marrow aspiration was repeated in early remission with informed consent for investigative purposes. Samples of marrow and blood were collected in sterile plastic tubes containing 10% vol/vol 0.14 M sodium citrate.

Cell processing

The marrow-citrate mixture was diluted in an equal volume of α medium (12) minus nucleosides ($\alpha-$). The diluted marrow and whole blood samples were layered atop an equal volume of a mixture containing 5.7 g Ficoll-400 and 8.0 g sodium diatrizoate/100 ml of water (Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, N. J.) and then centrifuged for 20 min at room temperature at 600 *g*. The layer of nucleated cells at the plasma-Ficoll-Paque interface was harvested, washed three times in $\alpha-$ medium and suspended in the $\alpha-$ medium at concentrations of $3–5 \times 10^7$ cells/ml for peripheral blood and $2–4 \times 10^6$ cells/ml for marrow. Almost all cells harvested from peripheral blood were lymphocytes and monocytes. There were no nucleated erythroid precursors and rare granulocytes. Cells harvested from marrow represented the full range of marrow nucleated precursors observed in direct marrow smears except for a relative deficiency of mature nucleated polychromatophilic and orthochromatophilic erythroid precursors and mature granulocytes.

Culture methods and quantification

Samples of 0.1 ml of cell suspension were added to 0.8 ml of the erythropoietin-dependent plasma clot incubation system described by McLeod and co-workers (13) as modified by Clarke and Housman (7). The final cell concentrations per milliliter of clot were $3–5 \times 10^6$ cells/ml for blood cells and $2–4 \times 10^5$ cells/ml of clot for marrow cells. Erythropoietin was derived from human urine and partially purified by Dr. Peter Dukes to a specific activity of approximately 50 IU/mg of protein.[2] Unless otherwise stated, studies of marrow CFU-E and BFU-E were performed at erythropoietin concentrations that produce maximal colony numbers in normal individuals (2–4 IU of erythropoietin/ml). Studies of blood BFU-E colony formation were performed at 4 IU/ml. In the initial assays, clotting was induced with beef embryo extract. This technique

[2] Kindly provided by Dr. Anne Ball, Blood Diseases Branch, Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute, Bethesda, Md.

TABLE I

Clinical Data at the Time of Studies of Peripheral Blood and Bone Marrow of 11 Patients with CHA in Relapse and in Remission

Case number	Patient	Age	Peripheral blood		Percentage of bone marrow nucleated cells		On steroids at time of study	Anemia responsive to steroids	Status of anemia at time of study
			Hemoglobin	Reticulocytes	Proerythroblasts	Total erythroid			
		yr	*g/100 ml*	*%*					
1	D. W.	1	6.0	0.6	8	9	No	—	Relapse
			7.5	1.4	—		Yes	—	Remission
			10.0	1.2	—		Yes	Yes	Remission
			8.2	0.8	—		Yes	—	Remission
2	B. R.	3	5.1	0.2	7	8	No	—	Relapse
3	K. G.	9	5.1	0.5	(a) 7	9	No	No	Relapse
					(b) 6	8	No	No	Relapse
4	M. C.	5	3.7	0	(a) 10	14	No	Partial	Relapse
			11.0	0	(b) 2	5	No	Partial	Relapse
			6.5	0.4	(c) 8	25	Yes	Partial	Relapse
5	J. S.	14	5.1	3.0	2	2	No	No	Relapse
6	T. B.	3	7.3	0	0	0	No	No	Relapse
7	L. F.	23	5.0	7.2	(a) 26	71	No	Yes	Relapse
			11.9	2.9	(b) 5	34	Yes	Yes	Remission
8	S. R.	17	10.4	2.4	—		Yes		Remission
			10.1	2.4	—		Yes	Yes	Remission
			9.7	1.4	—		Yes		Remission
9	J. O.	13	12.6	1.8	—		Yes	Yes	Remission
			12.6	—	—		Yes		Remission
10	N. C.	28	11.0	4.4	—		No	Yes	Remission
11	M. L.	6	9.2	2.4	—		Yes	Yes	Remission
Normal values			11–15	0.2–2.5	1–3	20–40			

The terms "partial response" and "remission" are explained in the Introduction. Study *b* of M. C. (patient 4) was performed after transfusion. In certain cases, studies of peripheral blood BFU-E were repeated one or more times while the patients were in remission.

proved unsatisfactory, particularly for growth of BFU-E colonies. Therefore, in the remaining studies, clotting was induced with 0.1 ml of NCTC 109 (Microbiological Associates, Bethesda, Md.) containing 1 U of grade 1 bovine thrombin (Sigma Chemical Co., St. Louis, Mo.). This was added to 0.9 ml of the cell incubation system. 0.10-ml samples of the clotting mixture were then rapidly dispensed into 0.2-ml microtitre culture wells (Linbro Plates, Linbro Chemical Co., New Haven, Conn.) and incubated under 5% CO_2 in high humidity. Under these conditions both BFU-E- and CFU-E-derived colonies grew satisfactorily.

The plasma clots were incubated for up to 14 days. On days 7, 10, and 14, and in some cases on intermediate days, the clots were fixed and stained as described by McLeod and co-workers (13). CFU-E were enumerated on day 7 by counting all of the benzidine-positive erythroid colonies of four cells or larger. BFU-E were scored on day 14 in marrow specimens and on intermediate days as well in blood specimens. The criteria for BFU-E were those described by Clarke and Houseman (7). Complete hemoglobinization of all subcolonies was not a requirement for inclusion as a BFU-E colony. For each determination of the number of BFU-E or CFU-E, two to three clots from each of a duplicate set of incubation mixtures were counted, and the mean scores and standard errors of the means of the four to six counts determined.

Evaluation of suppressor cells

To evaluate the possibility that certain cells in the peripheral blood of patients with CHA might suppress the growth of normal erythroid precursors (11), various mixtures of CHA peripheral blood nucleated cells with normal peripheral blood and marrow nucleated cells were made and incubated for varying periods of time. The particulars of these experiments are described in Results.

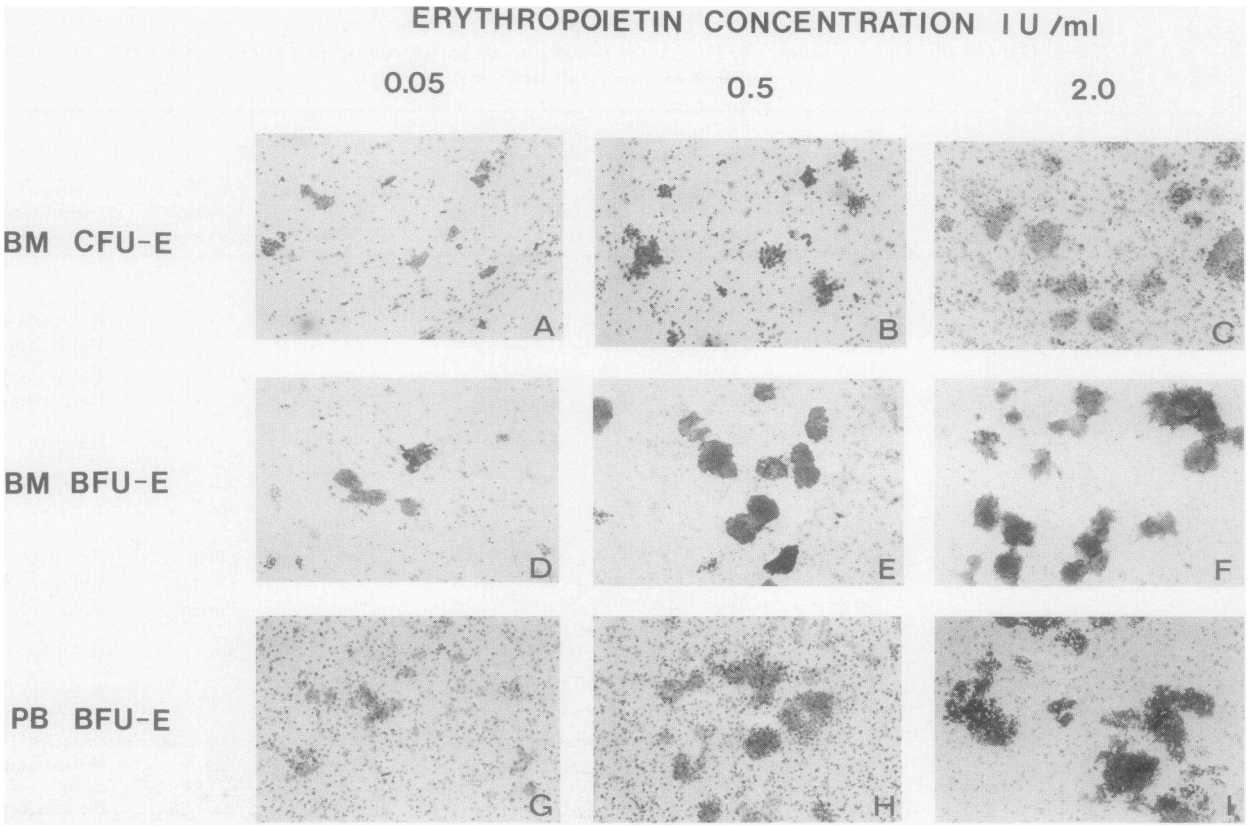

FIGURE 1 Photomicrographs that depict the influence of erythropoietin concentration on growth of erythroid precursors. See text for further description. BM, bone marrow. PB, peripheral blood.

RESULTS

BFU-E and CFU-E in normal and CHA peripheral blood and bone marrow

Morphology of normal CFU-E and BFU-E. Typical photomicrographs of human marrow CFU-E observed in cultures grown for 7 days in varying erythropoietin concentrations are shown in Fig. 1 A–C. There were no erythroid colonies when erythropoietin was not added. The size of the CFU-E clearly increased from tiny four-cell colonies to greater than thirty-two-cell colonies as the erythropoietin concentration was increased. At 2 U of erythropoietin, the mean CFU-E colony size was 16 cells. After 10 days of culture, most of the individual CFU-E colonies shown in Fig. 1 A–C had lysed, but recognizable early BFU-E colonies had begun to be evident. By day 14, they assumed the typical appearance of BFU-colonies as shown in Fig. 1 D–F. Note that the size and number of individual subcolonies within each BFU-E colony was greatly increased by higher erythropoietin concentrations. Representative blood BFU-E colonies are shown in Fig. 1 G–I. CFU-E were not detected in blood.

Effect of normal cell input on colony numbers. A linear relationship was observed between bone marrow cell input numbers and both BFU-E and CFU-E colony number in a range between 10^5 and 4×10^5 cells/ml (Fig. 2). All subsequent experiments with marrow were performed in this range. A linear range of BFU-E colony number in relation to cell input number has been previously demonstrated for blood cells (7), and all work with blood cells was performed within 10^6–4×10^6 cells/ml.

Enumeration of CFU-E and BFU-E in normal and congenital hypoplastic marrow and blood. The number of blood and marrow BFU-E and CFU-E colonies in normal subjects is set out in Fig. 3. The normal range was very wide. The inclusion of four-cell colonies in the CFU-E colony count increases the CFU-E score. At 2 U of erythropoietin/ml approximately 10% of the CFU-E colonies are four-cell colonies. Some of the variation in erythroid colony number per 10^5 marrow cells is probably influenced by the number of peripheral blood nucleated cells that contaminate a putative marrow aspirate because peripheral blood almost entirely lacks CFU-E and

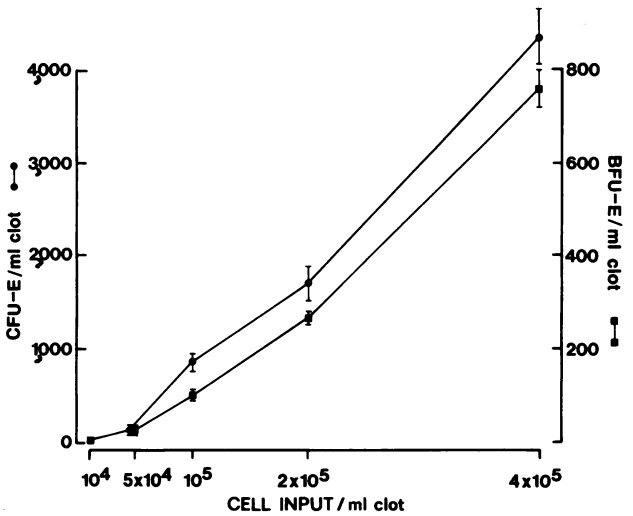

FIGURE 2 Relationships of marrow cell input to the number of CFU-E (●) and BFU-E (■) per milliliter of clot at 4 IU of erythropoietin/ml of clot. Each point represents the mean and SEM of the scores.

contains far fewer BFU-E than marrow. Estimates of the total nucleated cells in marrow (14), the total circulating lymphocytes and monocytes in peripheral blood, and the number of BFU-E per 10^5 marrow or blood nucleated cells lead to the conclusion that only approximately 1% of the total BFU-E circulate in peripheral blood.

Fig. 3 shows that there was usually a profound reduction of CFU-E in the marrows of relapsed CHA patients. One patient had a nearly normal number of CFU-E, but that was when the patient was severly anemic, at which time proerythroblasts and even more mature erythroid precursors were relatively increased in her marrow. After transfusion of red cells, marrow proerythroblasts and CFU-E were greatly reduced. However, we could not conclude that the number of proerythroblasts in the marrow samples of CHA patients were in general proportional to the number of CFU-E colonies observed. This was emphasized by cases 1, 2, 3, 4 c, and 7 in whom increased relative numbers of proerythroblasts were observed but CFU-E were deficient. Though CFU-E were occasionally present but decreased, BFU-E colonies were regularly absent from the marrows and peripheral blood cultures of all but one of the relapsed patients (case 7), in whom the number of BFU-E colonies was markedly reduced. A study of peripheral blood BFU-E colony formation in CHA in both relapse and in remission is

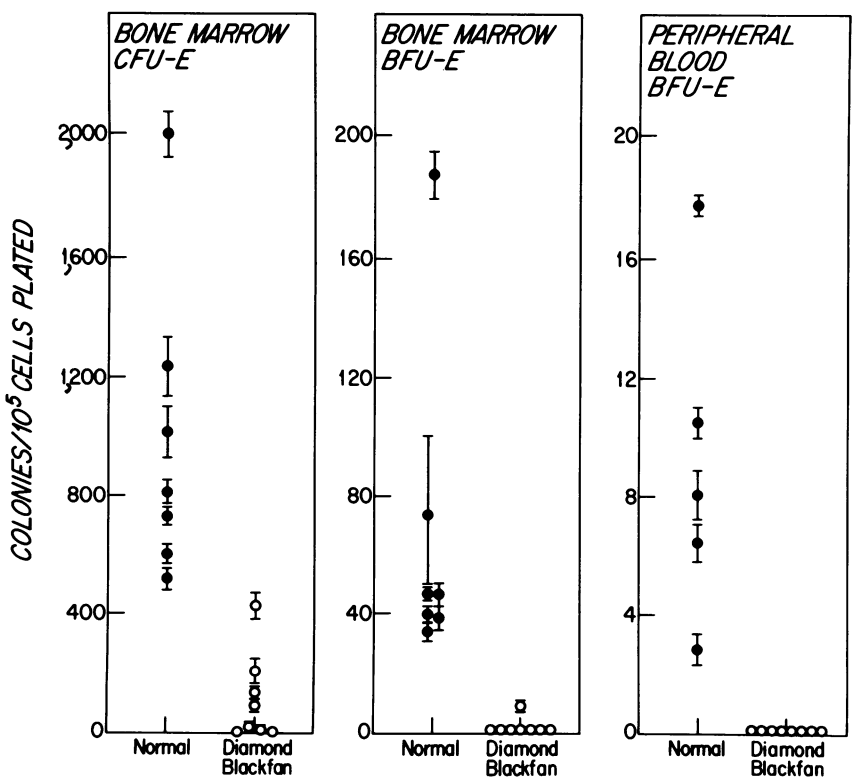

FIGURE 3 Comparison of the means and SEM of erythroid precursors in normal individuals (●) and patients with Diamond-Blackfan anemia (○) at 4 IU of erythropoietin/ml of clot. Each point represents the mean of a single study. A normal control was studied with each patient.

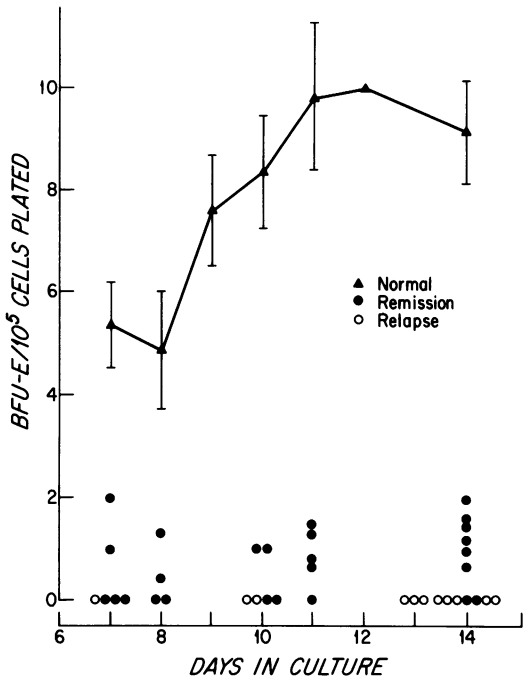

FIGURE 4 Rate of appearance of BFU-E during culture of peripheral blood mononuclear cells from normals (▲) and from patients with Diamond-Blackfan anemia in relapse (○), and in remission (●). For clarity, the SEM of the Diamond-Blackfan studies are not shown. They range from 10 to 25% of the mean.

shown in Fig. 4. Here the appearance of peripheral blood BFU-E colonies is presented as a function of time in culture. A gradual rise in the number of normal BFU-E colonies was observed. All relapsed cases of CHA exhibited complete absence of BFU-E in peripheral blood. Even after steroid-induced remission, the numbers of BFU-E colonies in peripheral blood were remarkably low, indicating persistence of this deficient state in the chronic disorder. The patients also failed to demonstrate an increase in BFU-E with time in culture.

Effects of erythropoietin concentration on CFU-E formation in CHA

Studies 7 a and 4 c provided an opportunity to evaluate the influence of the concentration of erythropoietin on CFU-E formation in relapse and, in study 7b, after steroid-induced remission. Though the number of CFU-E induced by 2–4 IU of erythropoietin was reduced in these patients, enough colonies were produced to examine the influence of lower erythropoietin concentrations on CFU-E colony formation. Fig. 5 demonstrates that the concentration of erythropoietin (approximately 1 IU/ml) required for half maximal stimulation of CFU-E colony number in these cases was 10 times greater than that required for normal CFU-E (0.1 IU/ml). In addition, the mean size of the CHA CFU-E colonies was much smaller than the normal CFU-E colonies at all erythropoietin concentrations. Additional comparisons of the erythropoietin sensitivity of CHA CFU-E were made with the marrow cells of three other normal individuals, a patient with hereditary spherocytosis and a patient with erythroid regeneration following aplastic anemia. In the latter conditions, as in normals, the CFU-E were 5–10 times more sensitive to erythropoietin than were CHA CFU-E (data not shown). Case 7 responded completely to steroid treatment. During this steroid-induced remission, the erythropoietin concentration dose-response curve shifted toward normal hormone sensitivity.

Effects of CHA blood cells on normal marrow CFU-E and blood BFU-E

In an attempt to investigate an immunologic basis for the deficiency of CFU-E in the bone marrow and BFU-E in the blood of patients with CHA, modifications of the studies of Hoffman and his co-workers (11) were performed. In the first of these studies, Ficoll-Paque-separated peripheral blood lymphocytes and monocytes of CHA patients were mixed in plasma clots with similar cells from normal individuals at concentrations of 10 and 50% of the total cell mixture. The cells of normal individuals with the widest possible range of BFU-E/10^5 peripheral blood cells were

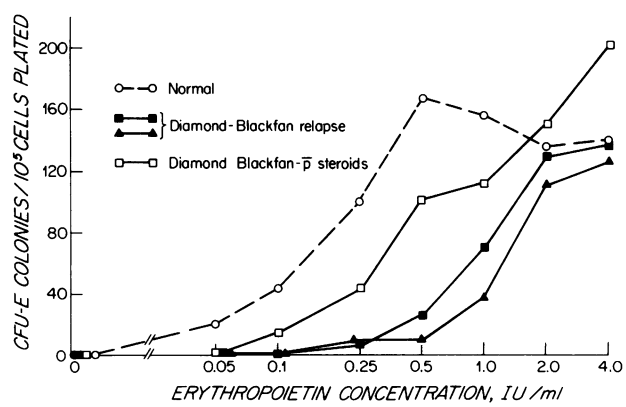

FIGURE 5 Growth of marrow CFU-E as a function of erythropoietin concentration in the culture medium. The normal marrow cells (○) were deliberately diluted in isologous peripheral blood nucleated cells in order to reduce the maximum CFU-E number to those observed in two relapsed patients with Diamond-Blackfan anemia (■, ▲). This dilution of normal marrow does not affect the shape of the dose-response curve (data not shown). After steroid-induced remission, the erythropoietin dose-response curve on one of the patients was repeated (□). Standard errors range from 5 to 15% of the means.

TABLE II

Effects of 10 and 50% Mixtures of CHA Peripheral Blood Nucleated Cells on Growth of BFU-E in Cultured Normal Peripheral Blood Nucleated Cells

Patient study	BFU-E colonies per 10^5 cells plated: Patient PB*	BFU-E colonies per 10^5 normal cells plated: Normal PB	Normal PB / Patient PB = 9/1	Normal PB / Patient PB = 1/1
1	0	15±2.5	13±2.8	20±4.3
3	0	2.6±0.6	2.0±0.8	2.6±0.3
4	0	10±1.7	8±0.3	6.4±1.5
6	0	4±0.7	3±0.2	4±1.1
8	0	4±0.3	5.6±1.5	4±1.1
9	0	18±3.1	12±2.1	20±1.6
10	1	10±0.8	12±0.7	16±2.7
11	0	3.4±0.9	3.0±0.8	4.6±1.1

The results in the last three columns are expressed as the mean±SEM of BFU-E colonies per 10^5 normal cells plated.
* Peripheral blood nucleated cells.

chosen as target cells, and CHA cells with absent BFU-E were used as effector cells. Peripheral blood BFU-E formation was then determined as shown in Table II. Inhibition of normal blood BFU-E formation did not occur when CHA peripheral blood nucleated cells were present in the mixture.

In the second of these co-culture studies, the peripheral blood nucleated cells of CHA patients who were either partially responsive or unresponsive to steroid therapy were mixed in plasma clots with normal marrow nucleated cells that had also been separated by Ficoll-Paque centrifugation. Two types of mixing studies were performed. In one study (Table III), the CHA cells were directly mixed and co-cultivated with normal marrow cells at 10, 33, and 50% of the total cell mixture. In another approach (Table IV) CHA peripheral blood nucleated cells were mixed with normal bone marrow cells at a ratio of 20:1. The marrow and peripheral blood cells were centrifuged for 10 min at 300 *g* to form a closely packed pellet. The pellet was then incubated at 37°C for 2–4 h in $\alpha-$ medium with 2% fetal calf serum before the cells were resuspended and cultured in the plasma clot system. Although in one study (involving patient 5) the growth of the normal control CFU-E colonies appeared to be inhibited by the manipulations, no evidence of a cytotoxic effect of CHA peripheral nucleated cells on normal CFU-E formation was developed in any of these studies. Nor was evidence gained to suggest that normal peripheral blood cells could correct the defect in CHA marrow cells.

DISCUSSION

The BFU-E was first discovered in murine marrow by Axelrad (2) and confirmed by Iscove and co-workers (3). Separation of murine marrow cells by unit gravity sedimentation has shown that BFU-E colonies appear later in marrow cultures than do CFU-E and because BFU-E have sufficient proliferative capacity to produce multiple CFU-E-like subcolonies, the BFU-E is thought to represent the first measurable commitment of the totipotential hematopoietic stem cell (CFU-S) to erythroid differentiation (2, 15). CFU-E, the daughter cells of BFU-E, probably represent a later stage in the stepwise differentiation toward erythrocyte production. Erythropoietin influences murine erythropoiesis from the BFU-E to the reticulocyte stage (16). A possible influence of the hormone on the differentiation of CFU-S to BFU-E has not been established.

TABLE III

Effects of 10, 33, and 50% Mixtures of CHA Peripheral Blood Nucleated Cells on the Growth of CFU-E in Cultured Normal Marrow

Patient study	CFU-E colonies per 10^5 normal BM cells plated: Normal BM*	Normal BM / Patient PB‡ = 9/1	Normal BM / Patient PB = 2/1	Normal BM / Patient PB = 1/1
3	185±43	222±36	184±21	235±18
4	1,154±124	1,172±103	ND	ND
5	800±64	813±97	970±114	810±72
6	520±27	693±53	804±103	578±87

The peripheral blood and marrow cells were mixed immediately before co-culture. The results are expressed as the mean±SEM of the number of CFU-E colonies per 10^5 normal marrow cells plated.
* Bone marrow.
‡ Peripheral blood nucleated cells.

TABLE IV

Effects of Peripheral Blood Cells on the Growth of Normal and CHA Bone Marrow CFU-E

Patient study	CFU-E colonies per 10^5 normal BM or Diamond-Blackfan BM cells plated			
	Normal PB / Normal BM	Diamond-Blackfan PB / Normal BM	Diamond-Blackfan PB / Diamond-Blackfan BM	Normal PB / Diamond-Blackfan BM
4	1,530±123	1,081±90	184±34	92±36
5	100±153	110±87	18±4	17±19
6	548±68	485±28	15±13	49±33

The ratio of peripheral blood to bone marrow as 20:1 and the blood and marrow cells were preincubated in a pellet for 2–4 h before co-culture. See text for details of these experiments. The results are expressed as the mean±SEM of the number of CFU-E colonies per 10^5 marrow cells plated.

More recently, the methylcellulose and plasma clot culture systems have been used for the analysis of normal erythropoiesis in human marrow. Tepperman et al. (6) suggested that both CFU-E and BFU-E could be observed in human marrow cultures after 7 days of incubation. Clarke and Housman (7) and Ogawa and co-workers (8) subsequently noted typical BFU-E colonies after 10- to 14-day culture of human nucleated blood cells. These typical clusters of subcolonies have also been observed in human marrow by Gregory (5) who has noted that BFU-E appear to vary with respect to total numbers of constituent subcolonies and the number of hemoglobinized cells in each of the subcolonies. The clonal origin of the multiple subcolonies within the BFU-E colony derived from human peripheral blood has been established by fluorescent antibody analysis of the hemoglobins in the individual subcolonies in cultures derived from mixtures of the nucleated cells of individuals homozygous for hemoglobins S and C (17). In our hands, the human marrow BFU-E population varies from 5 to 20% of the number of CFU-E, a ratio consistent with the precursor-progeny relationship postulated for these cells.

We find that both CFU-E and particularly BFU-E are markedly diminished as functioning units in the marrow and blood of relapsed patients with CHA, and functioning BFU-E are reduced in the peripheral blood of such patients even in partial remission. Freedman and Saunders (18) have recently suggested that CFU-E may not be deficient or defective in some cases of Diamond-Blackfan anemia because they have observed "normal" numbers of CFU-E colonies when 4×10^7 CHA marrow cells were separated into fractions in albumin gradients. But calculation of the recovery of colonies achieved by these investigators from data presented but not yet published reveals such large losses of normal CFU-E (from 125 CFU-E per 10^5 total bone marrow cells to approximately 6 CFU-E per 10^5 fractionated marrow cells) that their failure to observe significant differences between normal and Diamond-Blackfan marrows is of uncertain interpretation.

Because the deficiency of BFU-E function was so severe in the cases of CHA reported here, we were unable to perform erythropoietin dose-response curves in this disorder and compare these curves to the response of normal BFU-E to the hormone. However, studies 7 a and 4 c produced sufficient numbers of CFU-E to permit a comparison at this further differentiated step. The CFU-E in these cases were markedly insensitive to erythropoietin. The hormone concentration required for half-maximal CFU-E colony formation was 10 times higher than that required for normal CFU-E formation or for the growth of CFU-E in regenerating erythroid marrows. Thus the decreased sensitivity does not appear to be due to chronic exposure of the precursors to the high circulating levels of erythropoietin that occur in CHA. This point is relevant because Gregory and her co-workers have observed a two- or threefold decrease in the erythropoietin responsiveness of regenerating murine CFU-E in comparison to normal murine CFU-E (19). In one case (7 b), steroid therapy shifted the sensitivity of CHA CFU-E to erythropoietin toward normal. The reduced sensitivity of CHA CFU-E to erythropoietin toward normal. The reduced sensitivity of CHA CFU-E to erythropoietin and the apparent enhancement of sensitivity after steroids has several possible explanations.

Though these erythroid cultures are notoriously sensitive to minor changes in incubation conditions, the findings are not likely to represent artifacts of culture technique because normal control cultures were performed simultaneously. Obviously, a challenging explanation for this finding is that the abnormal curves represent a functional disturbance of putative erythropoietin receptors on the surface of the CHA erythroid precursor so that the affinity of this

abnormal cell for erythropoietin is diminished. Steroids might favorably alter that receptor and improve its capacity to interact with the hormone. Only the availability of highly purified and labeled erythropoietin (now just beginning to become available [20]) will permit an appropriate evaluation of this possibility. Meanwhile, it must be emphasized that erythropoietin dose-response curves are very complex experiments. The hormone preparation is itself highly impure. If the impure preparation is present in sufficiently high concentrations in the medium, it often suppresses CFU-E and BFU-E formation. Furthermore, the growth of CFU-E and BFU-E in such cultures does not in all likelihood represent the result of a simple interaction of hormone and precursor. Environmental factors such as cell-cell interactions that create certain helper molecules and perhaps suppressor effects undoubtedly play an important role (21). Many hormones enhance the growth of colonies in such cultures (22). Their sites of action remain uncertain. Thus, though we offer the suggestion that CHA represents a defect in the erythropoietin receptor system, further studies with materials not yet available will be necessary to explore this interesting possibility. That CHA might represent a structural disturbance of a protein such as a receptor suggests that the disease might have a genetic basis rather than represent a spontaneous mutation in all cases. In fact, we are becoming increasingly aware of the possibility of an inherited basis for this disorder. First, the disease is often accompanied by mesenchymal abnormalities similar to the type observed in the Fanconi anemia (23). Second, multiple cases have been reported in single families (24) and finally evidence for subtle abnormalities in erythropoiesis have been detected in the parents of some patients (25). A confounding characteristic of the disease is its disappearance after several years in some patients. This clinical fact does not suggest a structural defect in a single protein. Perhaps, however, unknown changes in the internal environment may affect the receptor in a manner similar to low doses of corticosteroids.

Because CFU-E are the products of BFU-E and some CFU-E are evident in most CHA marrows, the capacity of CFU-S to produce erythropoietin-responsive BFU-E cannot be entirely defective in this disorder. In fact, BFU-E *were* observed in the marrow of relapsed patient 7. It seems likely that under the influence of the high circulating erythropoietin levels characteristic of this anemia, the few responsive BFU-E that are generated differentiate to CFU-E and are not, therefore, detectable as BFU-E in the marrow. They "die" by differentiation as emphasized by Till and co-workers (26). That the number of early precursors in the marrow or blood represents in part their flux through differentiating compartments rather than their production alone is also exemplified by the fact that CFU-C may actually be reduced in the marrows of patients with marked increased granulocyte production and turnover (27).

A curious feature of many CHA marrows is the presence of nearly normal or even increased numbers of proerythroblasts without further erythroid differentiation. The CHA proerythroblasts probably share the unresponsiveness to erythropoietin that characterizes most of the BFU-E and CFU-E produced by the CHA CFU-S. Hence, these proerythroblasts fail to differentiate except in some severely anemic cases in which partial differentiation may occur in response to a sustained massive erythropoietin stimulus. Though produced in low numbers from a defective precursor pool, they accumulate at a stage where their development is arrested.

Hoffman and co-workers (11) have proposed an immunologic basis for CHA. They suggest that the disease is the result of suppression of CFU-E colony formation by abnormal CHA lymphocytes. We have found no evidence for an immunologic etiology in our patients. Four of the patients reported here were partially or totally resistent to steroid therapy but in none of them could we detect peripheral blood cells capable of preventing the development of normal marrow CFU-E or blood BFU-E colonies. However, it must be emphasized that technical differences may play an important role in such studies. For example, our marrow and blood mononuclear cell isolation methods and our plasma clot systems differ from those utilized by Hoffman and his co-workers. There may be a subset of patients with CHA and other forms of marrow failure in whom lymphocytotoxicity is present. That we did not detect this phenomenon in this study suggests that such lymphocytotoxicity is either unusual or difficult to demonstrate. Certainly subtle effects would be impossible for us to define given the observed inherent variation in colony growth. Finally, artifacts resulting from sensitization to histocompatibility antigens must always be considered in such allogeneic co-culture studies (28).

In summary, we currently propose that the abnormality in CHA is due to a qualitative or quantitative defect in the production of BFU-E from CFU-S. Our data cannot discriminate between these two possibilities. If BFU-E are produced, they or their environment must be relatively unresponsive to erythropoietin. They give rise to dysfunctioning CFU-E and, thence, to the accumulation of abnormal proerythroblasts. The latter are frozen in this early developmental stage as a consequence of decreased erythropoietin responsiveness. This leads to the aregenerative anemia. Steroid hormones partially repair the developmental defect in a manner presently unknown, but we theorize that they may improve the sensitivity of

the defective CHA erythroid precursors themselves or their environment to erythropoietin.

CHA or the Diamond-Blackfan anemia may, therefore, represent a congenital abnormality of erythropoietin responsiveness that causes a functional, if not absolute, deficiency of erythroid precursors.

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