Defective Erythroid Progenitor Differentiation System in Congenital Hypoplastic (Diamond-Blackfan) Anemia

By Jeffrey M. Lipton, Michele Kudisch, Rachel Gross, and David G. Nathan

To explore the etiology of congenital hypoplastic or Diamond-Blackfan anemia (DBA) we investigated in vitro erythropoiesis in nine patients. Of the nine, seven were clinically responsive to prednisone. Four were infants evaluated at the time of diagnosis. Six were never or were only minimally transfused. Those for whom prednisone had been prescribed had discontinued the drug a minimum of five months prior to study. The bone marrows of these nine patients were compared with those of hematologically normal individuals and with those of four patients with transient erythroblastopenia of childhood (TEC) whose erythroid aplasia was as severe as that of the patients with DBA. Using the plasma clot semisolid culture technique to enumerate erythroid progenitors and to evaluate the growth characteristics of the colonies to which they give rise, we concluded that at the onset of DBA: (a) erythroid progenitor frequency does not correlate with the degree of anemia and erythroblastopenia; (b) erythroid progenitor differentiation may in some cases be abnormally insensitive to crude preparations of erythropoietin; and (c) progenitor erythropoietin insensitivity in vitro does not necessarily indicate prednisone insensitivity in vivo. Thus, DBA does not appear to be solely the result of deficient formation of erythroid progenitors but is, in addition, a disorder that is due to defective progenitor differentiation in vivo.

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response to prednisone in vivo. Whether these findings are the result of an intrinsic progenitor defect or some other abnormality in the system that regulates terminal erythroid differentiation remains to be determined.

MATERIALS AND METHODS

Patients. Bone marrow was obtained after informed consent was received from nine patients who fulfilled the diagnostic criteria for DBA. The volume of all bone marrow aspirates was ~2 mL. The cell count after Ficoll-Hypaque density centrifugation was in all cases >40 x 10^6 mononuclear cells per aspirate. Thus, contamination by peripheral blood mononuclear cells and dilution of progenitors by these cells was considered to be <10%. The timing of the studies with relationship to age, diagnosis, and treatment is shown in Table 1. Three of the steroid-responsive patients were initially studied at diagnosis, two were studied prior to administration of red cell transfusions, and one was studied after a single transfusion. A fourth responsive patient was evaluated 18 months after diagnosis. She first received a brief course of androgens, followed by prednisone therapy with no transfusions. Two of the three other steroid-responsive patients were periodically transfused for seven months and 28 months, respectively, prior to this study. The remaining responsive patient, the only adult studied in this investigation, was transfused initially at diagnosis and received transfusions at the time of reinstitution of prednisone therapy after multiple elective cessations over a 20-year period. Of two steroid-nonresponsive patients, one was studied initially at diagnosis and before she received a transfusion, whereas the other was transfused approximately monthly for the 12 months prior to evaluation. Of the five patients previously treated with prednisone, all had discontinued the drug for a minimum of five months prior to evaluation. In addition to the normal marrows with which these studies of DBA marrow were compared, we also evaluated the marrows of four patients with transient erythroblastopenia of childhood or TEC. The patients were 12, 16, and 23 years of age, respectively, prior to this study. The remaining responsive patient, the only adult studied in this investigation, was transfused initially at diagnosis and received transfusions at the time of reinstitution of prednisone therapy after multiple elective cessations over a 20-year period. Of two steroid-nonresponsive patients, one was studied initially at diagnosis and before she received a transfusion, whereas the other was transfused approximately monthly for the 12 months prior to evaluation. Of the five patients previously treated with prednisone, all had discontinued the drug for a minimum of five months prior to evaluation. In addition to the normal marrows with which these studies of DBA marrow were compared, we also evaluated the marrows of four patients with transient erythroblastopenia of childhood or TEC. The patients were 12, 16, and 23 months of age, respectively. They all had reticulocyte counts of <1.0% with hemoglobins of 4.0, 4.3, 6.1, and 4.0 g/dL, respectively. Spontaneous recovery of erythrocytosis occurred within one month of evaluation of all four cases.

Cell culture. Bone marrow low-density mononuclear cells prepared by Ficoll-Hypaque density centrifugation were plated at a cell density of 5 x 10^3/mL in the plasma clot culture system previously described and modified. The culture system contained from 0.1 U/mL to 4 U/mL of erythropoietin from various sources. In some experiments, burst-promoting activity (BPA), the supernatant of the Mo cell line kindly provided by Dr David Golde, was substituted for NCTC-109 (Microbiological Associates, Bethesda, Md) as 5% of the plasma clot culture. We find this source of crude BPA to be comparable to that derived from leukocyte-conditioned medium.

Clotting was initiated by the addition of 0.1 mL of NCTC-109 containing 1 U of grade 1 bovine thrombin (Sigma Chemical Co, St Louis). The 1.0 mL of clotting mixture was dispensed in 0.1 mL aliquots into 0.2 mL microtiter culture wells (Linbro plates, Linbro Scientific Co, New Haven, Conn.) and incubated under 5% CO2 in high humidity. The plasma clots were incubated for up to 14 days. The clots were fixed and stained as described by McLeod and co-workers. Erythroid colonies in three to six clots were counted, and the results were expressed as the mean and SEM of the number of erythroid colonies per 1 x 10^6 cells plated. CFU-E were enumerated on day 7, and BFU-E were enumerated and scored according to size from 1+ to 4+ on days 11 through 14 as previously described.

Control marrows were obtained from hematologically normal individuals from three months of age to 50 years of age. Two were less than one year of age. The control studies were carried out during the time period in which this study was performed, frequently simultaneously, using identical tissue culture techniques.

RESULTS

Progenitor-derived colony enumeration. Previous data acquired in patients with long-standing DBA, of whom half were steroid resistant, suggested that the disorder in this

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Diagnosis</th>
<th>Age at Time of Study</th>
<th>Peripheral Blood at Presentation</th>
<th>Bone Marrow Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hemoglobin (g/100 mL)</td>
<td>Reticulocytes (%)</td>
</tr>
<tr>
<td>E.D.</td>
<td>2 mo</td>
<td>2 mo</td>
<td>8.3</td>
<td>0.6</td>
</tr>
<tr>
<td>M.H.</td>
<td>2 mo</td>
<td>2 mo</td>
<td>5.8</td>
<td>1.6</td>
</tr>
<tr>
<td>K.G.</td>
<td>3 mo</td>
<td>3 mo</td>
<td>3.9</td>
<td>1.5</td>
</tr>
<tr>
<td>J.F.</td>
<td>2 mo</td>
<td>1 yr, 8 mo</td>
<td>6.6</td>
<td>0.3</td>
</tr>
<tr>
<td>J.C.</td>
<td>1 mo</td>
<td>8 mo</td>
<td>5.3</td>
<td>0.0</td>
</tr>
<tr>
<td>H.S.</td>
<td>4 mo</td>
<td>2 yr, 8 mo–3 yr, 9 mo</td>
<td>3.0</td>
<td>0.2</td>
</tr>
<tr>
<td>P.M.</td>
<td>2 yr, 2 mo</td>
<td>23 yr</td>
<td>6.6</td>
<td>0.0</td>
</tr>
<tr>
<td>R.C.</td>
<td>1 mo</td>
<td>1 mo–1 yr, 10 mo</td>
<td>6.2</td>
<td>0.2</td>
</tr>
<tr>
<td>J.D.</td>
<td>2 mo</td>
<td>1 yr, 2 mo</td>
<td>2.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Although hematologically responsive to prednisone, untoward side effects such as linear growth retardation, weight gain, and hypertension necessitated prednisone withdrawal and transfusion dependence.

DBA, Diamond-Blackfan anemia; PE, proerythroblast; BN, basophilic normoblast; PN, polychromatophilic normoblast; ON, orthochromat normoblast; MT, multiple transfusions.

*The erythropoietin sources used in this study were: Sheep plasma, Connaught, Step II, Connaught Medical Research Laboratories, Willowdale, Ontario, Canada (Lot nos: 3029-1, 3031-1, 3032-1, 3039-1, 3042-1, 3076-1, and 3081-1), specific activity (sp. act.) 3 to 5 U/mg of protein; human urinary, National Heart, Lung and Blood Institute (NHLBI) (H-36-TaLSL), sp. act. 28 U/mg of protein; human urinary NHLBI (Arg-8-TaLSL), sp. act. 27 U/mg of protein; human urinary, NHLBI (E-8-39LSL), sp. act. 28 U/mg of protein; human urinary, Dr Arthur Sytkowski, Boston, sp. act. 96 U/mg of protein; and human urinary, Toyobo Co, Ltd, Osaka, Japan, (Lot no. 1668), sp. act. 49 U/mg of protein.
group is characterized by a block in progenitor maturation at some point between the multipotent myeloid progenitor and the BFU-E stage of differentiation. To evaluate this finding in the patients studied, erythroid progenitor-derived colonies were enumerated in plasma clot at 2 U/mL of erythropoietin. A degree of marrow erythroid aplasia far in excess of that predicted by the usual precursor product relationship of CFU-E to marrow erythroblasts provides strong evidence that in vivo progenitor maturation is relatively intact from the multipotent progenitor to the CFU-E stage of differentiation, and that the in vivo defect in DBA lies in the inability of CFU-E to differentiate beyond that stage. As shown in Fig 1, the mean and 1 SD of the frequency of CFU-E-derived colonies in DBA was lower than normal; however, normal frequencies were found in four of seven steroid-responsive patients. (The SEM for individual patients and controls was in the range of ±15% to 25%, in agreement with previous work using these culture techniques.) The progenitor frequencies in the three remaining steroid-responsive patients, although low, were far in excess of that expected for the degree of reticulocytopenia. The cultures of marrows of the two steroid-nonresponsive patients provided relatively poor colony growth. CFU-E colony expression in the steroid-nonresponsive patients fell below 1 SD of the mean for the nine DBA patients. However, addition of a source of BPA to the latter cultures significantly enhanced their CFU-E-derived colony expression, suggesting that CFU-E are present in the marrow in sufficient numbers but that, in contrast to the less severely affected steroid-responsive patient, standard culture techniques do not overcome the block in differentiation to hemoglobin-containing cells unless BPA is added. The addition of BPA enhanced CFU-E-derived colony numbers in all five of the DBA marrows to which it was added. In contrast, the frequency of CFU-E-derived colonies was enhanced by BPA only in one apparently normal marrow. The marrow from this individual provided the only “normal” study in which CFU-E-derived colony frequency, in the absence of BPA, was below the normal range. Measurements of BFU-E-derived colony frequencies provided similar results. Three of the seven steroid-responsive patients had normal colony frequencies. BFU-E colony frequency was markedly increased in two of these patients and was moderately increased in a third steroid-responsive patient in the presence of BPA. Neither of the steroid-nonresponsive patients produced normal BFU-E-derived colony frequencies, and colony size was persistently deficient (1+ instead of 3 to 4+) in five of the cultures of the marrows of steroid-responsive patients and both steroid-nonresponsive patients. The significant enhancement of CFU-E-derived colony expression even for prednisone-resistant patients in the presence of BPA and BFU-E-derived colonies that were considerably smaller than those derived from normal controls strongly suggest that the failure of erythropoiesis results primarily from dysfunction of the terminal differentiation of erythroid progenitors rather than from their absence.

Erythropoietin sensitivity of progenitor differentiation. There appears to be a complex system involving hematopoietic growth factors and cell–cell interactions that regulate the terminal differentiation of erythroid progenitors. To gain further evidence of dysfunction of this erythroid progenitor differentiation system, we measured the crude erythropoietin requirement for in vitro DBA progenitor differentiation. To do so, we enumerated progenitor-derived colonies as a function of the erythropoietin concentration in the cultures. From these data, an erythropoietin dose-response curve was generated (Fig 2), and the [Epo]_{1/2max}, that concentration of erythropoietin at which half the maximal number of progenitor-derived colonies are obtained, was determined. The abnormal dose-response curve shown in Fig 2 is one of two patient curves that do not approach a plateau level for erythroid progenitor-derived colony expression at or below an erythropoietin concentration of 4 U/mL. In this case, greater colony expression at a higher erythropoietin concentration would shift the [Epo]_{1/2max} to an even higher value. As summarized in Fig 3, of the seven steroid-responsive patients, two had an abnormally high [Epo]_{1/2max} for BFU-E and CFU-E indicative of

![Fig 1. Erythropoietin sensitivity of progenitor differentiation.](image)

![Fig 2. Representative erythropoietin dose-response curves in two patients with Diamond-Blackfan anemia (DBA). The percentage of maximal BFU-E colony expression on the vertical axis is plotted as a function of erythropoietin concentration from 0 to 4.0 U/mL on the horizontal axis for a patient with normal in vitro erythropoietin sensitivity (O—O) and a patient with low in vitro erythropoietin sensitivity (O—O). The concentration of erythropoietin at which there is 50% maximal BFU-E colony expression is defined as the half maximal erythropoietin concentration or [Epo]_{1/2max}.](image)
in vitro insensitivity to erythropoietin. Thus, five patients with steroid-responsive DBA had no evidence of a high erythropoietin requirement when compared with normal subjects and patients with TEC as described below. However, of these five, three had small (1 +) BFU-E-derived colonies at two units of erythropoietin per milliliter. Both of the steroid-nonresponsive patients had an elevated [Epo]/2max and small progenitor-derived colonies. Although evaluated in only two abnormal patients, the presence of BPA did not normalize the [Epo]/2max in steroid-resistant patients. In summary, an in vitro defect in the terminal differentiation of erythroid progenitors measured by an elevated [Epo]/2max could be demonstrated in two of seven prednisone-responsive and in both prednisone-nonresponsive patients. Five steroid-responsive patients had progenitor differentiation that exhibited a normal [Epo]/2max when compared with normal and TEC progenitors. Three of these patients and both steroid-resistant patients had small BFU-E-derived colonies. Two of the steroid-responsive patients exhibited normal erythropoietin sensitivity as measured by [Epo]/2max and colony size. Table 2 summarizes DBA progenitor-derived colony enumeration and erythropoietin dose response data.

Progenitor colony enumeration and erythropoietin sensitivity in transient erythroblastopenia of childhood (TEC). To determine whether the findings in DBA were merely due to some unexpected and ill-defined result of severe anemia and erythroid hypoplasia, we pursued similar studies in patients with TEC, a spontaneously resolving red cell aplasia which initially is frequently indistinguishable from DBA.2,16,17 Both syndromes are characterized by anemia, reticulocytopenia and elevated plasma erythropoietin activity, and the absence of erythroid precursors in the marrow, but TEC is not familial and there are no associated congenital abnormalities. Erythroid failure in many TEC patients appears to be due to an IgG inhibitor directed against erythroid progenitors.18,19 In agreement with the studies of Dessypris et al.,18 we confirm that progenitor colony enumeration in TEC yields variable results (Fig 1). One patient had normal CFU-E- and BFU-E-derived colony frequencies. Two patients had low numbers of CFU-E- and normal BFU-E-derived colony numbers, and a fourth TEC patient had low BFU-E and CFU-E colony frequencies. The presence of BPA had no effect on progenitor-derived colony expression in the one TEC patient so studied. The BFU-E [Epo]/2max values in the three TEC patients with normal BFU-E-derived colony numbers were within the normal range. BFU-E colony morphology was normal (4 +) in those three cases. The [Epo]/2max for BFU-E-derived colonies in one patient was elevated, indicative of erythropoietin insensitivity. BFU-E-derived colonies in this case were small (1 +). All TEC patients had mild to moderate elevations in [Epo]/2max for CFU-E-derived colonies. Thus, in the patients with TEC in whom anemia, reticulocytopenia, and bone marrow erythroid aplasia were comparable to that observed in DBA, erythroid progenitor behavior was different. BFU-E-derived colony frequency, morphology, and response to erythropoietin was normal in three of four patients, whereas CFU-E, although usually reduced in frequency, had an essentially normal in vitro response to erythropoietin. If one considers the minimal elevation in CFU-E [Epo]/2max in TEC to be in some way related to the degree of erythroblastopenia observed in both TEC and DBA, both steroid-nonresponsive and two steroid-responsive patients clearly have a progenitor differentiation abnormality as measured by an abnormal [Epo]/2max. These observations support the contention that the abnormal in vitro colony frequencies, morphology, and response to crude erythropoietin when observed in patients with DBA are actually due to defects in progenitor differentiation and are not caused by narrow erythroid hypoplasia and elevated plasma erythropoietin levels.
poietin levels. In addition, the presence of a markedly abnormal erythropoietin dose response in one TEC patient and low CFU-E colony frequency in three TEC patients, supports the observation of Dessypris et al19 that TEC is a heterogenous disorder which on occasion may mimic DBA.

DISCUSSION

Recent advances in experimental hematology have identified the existence of a pool of erythroid bone marrow progenitors which renew the morphologically identifiable precursor pool.20-23 The trilineage myeloid progenitor is a cell capable of giving rise to granulocyte/macrophage and megakaryocyte progenitors and to the least mature erythroid progenitor, BFU-E. Under ordinary physiologic conditions in vivo this primitive cell divides and matures to form the next stage of progenitor development, the erythroid colony forming unit or CFU-E. The exact number of erythroid progenitors in human marrow is unknown because they are enumerated only in functional assays. Their differentiation in culture to "normal" numbers of colonies has been thought to indicate the presence of normal numbers of functional progenitors. The development of BFU-E and CFU-E into erythroid colonies has a characteristic pattern of in vitro sensitivities to erythropoietin. The program of differentiation of normal CFU-E is more sensitive to the hormone than is the system that controls the differentiation of BFU-E.24,20-22

Failure of erythropoiesis could result from defects at any of the steps in differentiation from progenitor to reticulocyte. Enumeration of progenitor-derived colonies has therefore been used in an attempt to define the level of erythropoiesis that is inhibited in DBA. Nathan et al8 studied erythroid progenitor-derived colony numbers in a series of patients with DBA, most of whom had had the disease for at least five years and were either transfusion dependent or taking prednisone. None were evaluated at the time of diagnosis. The absence of detectable BFU-E in this group of patients led these investigators to conclude that the defect arises at an early step, the commitment of trilineage progenitors to BFU-E. Freedman and co-workers detected patients with normal as well as low numbers of CFU-E.17 BFU-E were not measured, and the relationship of the studies to the time of diagnosis was not specified. Variable results have been reported in smaller studies.9,24-26 Nathan et al8 and Chan et al9 also demonstrated that the progenitors of some patients with DBA exhibit relative erythropoietin insensitivity. In some of these cases, the erythropoietin dose response could be normalized somewhat by the administration of prednisone in vivo or even in vitro,4 suggesting to Chan and co-workers48 the possibility that the degree of erythropoietin insensitivity and the response to prednisone in vitro might correlate with a clinical steroid response. The exact mechanism of this in vitro abnormality in progenitor differentiation remains controversial. An intrinsic progenitor defect may be the case, but other workers have suggested that red cell failure in DBA is caused by the suppression of erythroid differentiation by cytotoxic or autoreactive T lymphocytes27,28 or by a bone marrow microenvironmental defect.24 In addition, other abnormalities of lymphocyte function have been observed in this disease.29 Abnormal lymphocyte function suggests that a suppressor mechanism may be operative. However, transfection sensitization could explain these results.30,31 Other investigators have shown that peripheral blood T cells from patients with DBA are able to induce in vitro erythropoietin-dependent BFU-E colony expression as do normal T cells22 and have no suppressor function.6,32 One group of investigators appears to have identified a serum factor that inhibits erythropoiesis in DBA,34 but further attempts to identify such humoral inhibitors have not been successful,7,24,35 in contrast to the findings in TEC.18,19

In this study, erythroid progenitor-derived colonies from patients with DBA were enumerated in cultures containing erythropoietin at a concentration of 2 U/mL. The mean colony frequency observed in DBA was lower than the mean of normal controls and the steroid-resistant patients had values in the lowest range, although the addition of BPA significantly enhanced their CFU-E colony expression. However, if one concludes that a significant frequency of committed progenitors, especially CFU-E, indicates a relatively intact pathway of differentiation from trilineage to committed progenitors, there does not appear to be an in vivo defect in progenitor maturation, especially in early cases of steroid-responsive DBA, that is significant enough solely to account for the observed degree of anemia and reticulocytopenia.

To determine the presence of a functional defect in the erythropoietin-dependent differentiation of DBA progenitors, both colony size and a progenitor erythropoietin dose response was evaluated in each patient studied. Results showed that seven of nine patients had an abnormality in the terminal differentiation of progenitors to colonies of hemoglobinized cells, characterized by these measures of insensitivity to erythropoietin. Although both patients who were refractory to prednisone therapy had abnormal dose responses to crude erythropoietin and small BFU-E-derived colonies, the presence of two responsive patients with identical in vitro abnormalities and three with small BFU-E-derived colonies but a normal erythropoietin dose response indicates that DBA is a heterogenous disorder in which abnormal progenitor differentiation in response to crude erythropoietin using the culture system we use is common. The variation in severity does not necessarily correlate with a lack of clinical responsiveness to prednisone therapy.

The study of bone marrow erythroid progenitors from patients with TEC provides an evaluation of progenitor frequency and differentiation in response to erythropoietin in the face of a level of anemia, reticulocytopenia, and bone marrow erythroid aplasia similar to that seen in DBA. In three of four patients studied, we were able to demonstrate normal BFU-E colony morphology and response to erythropoietin. CFU-E colonies were deficient in three of four TEC patients. The [Epo]/max in these cases was mildly elevated. The defect in two steroid-responsive and in both nonresponsive DBA patients was far in excess of this mild abnormality. We therefore conclude that the normal progenitor frequencies observed in DBA were not due to a concentration artifact and that the abnormal response to erythropoietin in DBA is not merely an epiphenomenon associated with a high degree of erythroid aplasia.
A large body of data suggests that the defect in erythroid differentiation in DBA is a consequence not of an intrinsic progenitor abnormality such as a decreased or abnormal responsiveness to erythropoietin and/or BPA, but of the extrinsic suppression of progenitor differentiation.\textsuperscript{24,27,28} Although it is possible that high levels of these mediators may overwhelm a suppressor mechanism, the requirement in severe cases for exogenous BPA to induce the terminal differentiation of DBA CFU-E in vitro and the relative insensitivity of the system to crude erythropoietin also suggest that there could be defective BPA production by DBA marrow accessory cells. The addition of high concentrations of crude erythropoietin may even enhance colony expression in less severe cases by providing exogenous BPA. Thus, the use of impure erythropoietin preparations containing BPA may have obscured an in vitro defect in the progenitor differentiation system in the steroid-responsive patients. Recently, we have been able to fractionate normal marrow into a highly enriched progenitor population and three distinct accessory cell populations.\textsuperscript{36} When this system was used, normal CFU-E removed from all sources of BPA have been shown to require small amounts of exogenous BPA to induce erythropoietin-dependent differentiation. The data presented in this study demonstrate that congenital hypoplastic or DBA is a heterogenous disorder. There appears to be no significant block in erythroid maturation from the trilineage myeloid progenitor to CFU-E. However, there is instead a defect in the terminal differentiation system of erythroid progenitors. This observation and further data demonstrating an abnormal response of some DBA progenitors to crude erythropoietin and BPA provide the essential framework for more definitive studies. We should now be able to examine the response of highly enriched progenitors to pure preparations of erythropoietin and BPA and to evaluate bone marrow accessory cell function in DBA. These studies, based on the data presented here, will allow for a more precise understanding of the apparently diverse pathophysiology of DBA.

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