Diamond-Blackfan Anemia: Promotion of Marrow Erythropoiesis In Vitro by Recombinant Interleukin-3

By Daniel S. Halperin, Zeev Estrov, and Melvin H. Freedman

To clarify the defective erythropoiesis in eight patients with Diamond-Blackfan anemia, we studied their bone marrow response in vitro to recombinant human interleukin-3 (IL-3) and recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). In an erythropoietin-containing assay system, specimens from six of the eight patients yielded low numbers of erythroid colonies compared to controls, and in five of these no erythropoietin dose-response could be elicited. Addition of IL-3, GM-CSF or both to cultures from the six patients had no effect on CFU-E-derived colonies. In contrast, IL-3 but not GM-CSF induced a marked increase in the number (183%) and size of the BFU-E-derived colonies in five of the six cases.

Diamond-Blackfan anemia is a congenital anomaly of erythropoiesis characterized by a normochromic macrocytic anemia of infancy, absent reticulocyte response, and a normally cellular marrow showing markedly decreased erythroid cells with normal myeloid and megakaryocytic maturation.1,2 Over 50% of cases initially respond to corticosteroids with a brisk wave of marrow erythropoiesis, but most patients are unable to maintain a stable, therapy-independent remission and eventually require long-term corticosteroids or regular red-cell transfusions.3 A few nonresponders to steroids have been successfully treated with alelogeic bone marrow transplants.4-6

Despite numerous studies, the pathogenesis of Diamond-Blackfan anemia has not been fully elucidated. Reports implicating a T-cell mediated inhibition7-10 or a humoral inhibitor11 have been contradicted by others,12,13 and the general consensus is that the fundamental defect lies at the level of the bone marrow erythroid progenitors, which demonstrate variable degrees of insensitivity to erythropoietin or are unable to differentiate to a complete stage of maturation.14,15

In this study, we used a novel approach to clarify the mechanism of defective erythropoiesis in this disorder. Since the recombinant human growth factors, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF), are both powerful stimulators of hematopoiesis, we studied their effects singly and in combination on the proliferation in vitro of two classes of bone marrow erythroid progenitors, termed CFU-E and BFU-E, from patients with Diamond-Blackfan anemia. Our data demonstrated that IL-3 but not GM-CSF enhanced marrow erythropoiesis in most patients studied, and exerted a corrective effect on the aberrant colony formation.

MATERIALS AND METHODS

Subjects. Eight patients (four boys and four girls) were studied (Table 1). All fit the classic clinical and hematologic description of Diamond-Blackfan anemia.1 All had normal karyotypes prepared from mitogen-stimulated peripheral blood lymphocytes. At the time of diagnosis and of this study, marrow aspirates from all showed normal cellularity with an isolated reduction of the red cell series in which proerythroblasts never exceeded 3% of the differential count. Although counts of total white cells, neutrophils, and platelets were normal in all patients at the time of diagnosis, values were slightly reduced in patient nos. 3 and 5 (Table 1) at the time of this study without explanation. In patient no. 5 the neutropenia was transient whereas in patient no. 3 there had been a slow progression over the previous 2 years towards leukopenia and thrombocytopenia.

Two patients had shown clinical response to prednisone in the past, but treatment was stopped because of its toxicity and no patients were on corticosteroids at the time of the study. All were on a monthly transfusion program, and seven of eight patients were on chelation treatment with daily subcutaneous deferoxamine infusions. Bone marrow was obtained immediately prior to transfusion in each case.

Control bone marrow for this study was aspirated from three healthy adult volunteers. Control marrow colony numbers for the pediatric age range were established previously on samples obtained from hematologically normal patients who underwent the procedure for diagnostic purposes. These studies were approved by the ethics committee of our institution and informed consent was obtained from all the patients and their families.

Colony-stimulating factors. Recombinant human IL-3 and GM-CSF were provided as a courtesy by Dr Steven C. Clark (Genetics Institute, Cambridge, MA). Supernatant of COS-1 cells transfected with human cDNA encoding for IL-3 was used as the source of IL-3,14 whereas GM-CSF was purified to homogeneity (>99%) from Chinese hamster ovary conditioned medium.20 In a proliferation assay using cells from patients with chronic myelogenous leukemia, the saturating bioactivity of IL-3 was obtained at a dilution of 1:2,500. The specific activity of GM-CSF was 8 x 10^6 U/mg protein. In our assay, IL-3 was used in dilutions of 1:10,000-1:1,250 (10^-4 to 8 x 10^-4) and GM-CSF at concentrations of 0.25-2 U/mL. A unit of GM-CSF was defined as the final

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concentration that gave one-half maximal colony growth in a
CFU-GEMM assay. In preliminary studies using various dilutions
of IL-3, optimal BFU-E colony growth was found with 1:5,000 and
optimal CFU-GM growth at 1:1,250. Both cytokines were added to
the cultures simultaneously with the other culture ingredients.

**Erythroid clonogenic assay.** The CFU-E/BFU-E assay was
performed with slight modifications of the method described by
Iscove et al. This assay was utilized because it profiles CFU-E,
BFU-E, and granulocytic-macrophage (CFU-GM) colony growth in
the same culture plate, and provides an optimal medium for
erythroid progenitor colony proliferation. Briefly, neutrophils and
red cells were removed from heparinized (10 U/mL) bone marrow
during centrifugation (800 × g at 4°C, 10 minutes) over Percoll (den-}
sity:1.077 g/mL; Pharmacia Fine Chemicals, Piscataway, NJ). After
Percoll separation, mononuclear bone marrow cells (1 × 10^7) were
suspended in a-medium, 30% fetal calf serum (Gibeo, Grand Island,
NY), 1% bovine serum albumin (Sigma Chemicals, St. Louis, MO)
and methylcellulose (Dow Chemicals, Midland, MI) at a final
concentration of 0.84% in 35 mm Petri dishes. Partly purified (25- to
30-fold) human erythropoietin (British Columbia Cancer Research
Centre, Vancouver), devoid of burst promoting activity at the
concentrations used, was added at final concentrations of 1-6
U/mL. The cultures were incubated at 37°C in air with 5% CO2 and
high humidity. Colonies were counted on day 7 (for CFU-E) and 14
(for BFU-E and CFU-GM) using an inverted microscope. A CFU-E
colony was defined as a cluster of eight or more hemoglobinized cells,
or three or more smaller subcolonies of 500 or more hemoglobinized cells, or three or more smaller subcolonies
of three or more smaller subcolonies of day 14. A CFU-GM colony was defined as a cluster of 20 or
more cells consisting of granulocytes, monocytes-macrophages, or
both. All studies were performed in duplicate.

**Statistical analysis.** The statistical probability of significant
differences between colony numbers in control and study experi-
ments was determined by Student's t test on paired independent
variables.

**RESULTS**

**Effect of IL-3 and GM-CSF on normal bone marrow**
**colony proliferation.** Cell cultures of morphologically nor-
mal bone marrow from three healthy adult volunteers were
initially used to evaluate the response of erythroid and
myeloid progenitors to IL-3 and GM-CSF for comparison
with the study group. No hemoglobinized erythroid colonies
were detected in the absence of erythropoietin. With erythro-
poietin, 2 U/mL, the marrows yielded mean CFU-E and
BFU-E colony numbers (± SD) of 238 ± 39 and 122 ± 41 per
10^3 cells plated, respectively (Table 2), and colony numbers
increased with increasing concentrations of erythropoietin up
to 4 U/mL with a slight decline, thereafter. In cultures containing a fixed dose of erythropoietin (2 U/mL), graded
centration of IL-3 (dilutions of 1:10,000 to 1:1,250),
GM-CSF (0.25 to 2.0 U/mL), or both, did not produce a

### Table 1. Clinical and Hematologic Data on Eight Patients with Diamond-Blackfan Anemia

<table>
<thead>
<tr>
<th>Patients</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
<td>Age at diagnosis (mo)</td>
<td>Birth</td>
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<td>1.5</td>
<td>Birth</td>
<td>3</td>
<td>3</td>
<td>Birth</td>
<td>1.5</td>
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<tr>
<td>Age at present study (yr)</td>
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<td>3.3</td>
<td>13</td>
<td>11.2</td>
<td>9.4</td>
<td>5.9</td>
<td>5.5</td>
<td>0.3</td>
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<td>Congenital anomalies</td>
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<td>b</td>
<td>c</td>
<td>d, e</td>
<td>e, f, g, h</td>
<td>i</td>
<td>None</td>
</tr>
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<td>Growth retardation</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Response to steroids</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Initial Hb (g/L)</td>
<td>59</td>
<td>33</td>
<td>40</td>
<td>77</td>
<td>18</td>
<td>19</td>
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<td>66</td>
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<td>MCV (μm²)</td>
<td>124</td>
<td>87</td>
<td>91</td>
<td>86 *</td>
<td>96</td>
<td>98</td>
<td>121</td>
<td>94</td>
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<td>WBC (×10⁹/L)</td>
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<td>14.9</td>
<td>6.3</td>
<td>6.2</td>
<td>6.9</td>
<td>7.2</td>
<td>8.2</td>
<td>7.2</td>
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<tr>
<td>ANC (×10⁹/L)</td>
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<td>2.4</td>
<td>2.9</td>
<td>1.8</td>
<td>3.5</td>
<td>2.9</td>
<td>3.4</td>
<td>1.5</td>
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<tr>
<td>Platelets (×10⁹/L)</td>
<td>160</td>
<td>503</td>
<td>245</td>
<td>340</td>
<td>350</td>
<td>270</td>
<td>325</td>
<td>538</td>
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<td>MCV determination post-transfusion.</td>
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</table>

Abbreviations: Hb, hemoglobin; WBC, white blood cell count; ANC, absolute neutrophil count. Anomalies: a, congenital glaucoma; b, double ureter; c, bifid thumb; d, undescended testes; e, growth failure; f, triphalangeal thumb; g, subluxated hips; h, dysmorphic facies; i, abnormal humerus.

*MCV determination post-transfusion.

### Table 2. Erythroid and Granulocyte-Macrophage Colony Formation Induced by IL-3 and GM-CSF in Cultures of Three Control Bone Marrows

| Colonies Per 10⁶ Cells Plated* (Mean ± SD) | | | | | | | |
|------------------------------------------|---|---|---|---|---|---|---|---|
| EPO (2 U/mL) | 238 ± 39 | 122 ± 41 | 124 ± 36 |
| EPO (2 U/mL) + IL-3† | 241 ± 56 | 140 ± 44 | 15% | 352 ± 78 | 184% |
| EPO (2 U/mL) + GM-CSF (1 U/mL) | 222 ± 31 | 131 ± 68 | 7% | 392 ± 92 | 216% |
| EPO (2 U/mL) + IL-3† + GM-CSF (1 U/mL) | 213 ± 49 | 139 ± 49 | 14% | 423 ± 77 | 241% |

*Mean number of colonies from duplicate cultures of 3 bone marrow samples.
†For the data shown in this Table, a dilution of 1:5,000 of IL-3 was used for erythroid colony studies and a dilution of 1:1,250 for the CFU-GM studies.
change in colony numbers. Neither IL-3 nor GM-CSF when used alone or in combination was able to promote erythroid colony growth in absence of erythropoietin.

Using standard culture conditions in the absence of IL-3 and GM-CSF, the control marrows yielded mean (±SD) CFU-GM colony numbers of 124 ± 36 per 10^5 cells plated. By adding IL-3, GM-CSF or both, sharp increases in CFU-GM colonies were observed in all studies with the highest numbers in cultures containing both growth factors (Table 2).

The baseline colony numbers for CFU-E, BFU-E, and CFU-GM from the three control marrows were within the range of control values established in our laboratory on hematologically normal specimens.

**Effect of IL-3 and GM-CSF on bone marrow colony growth from patients.** Using standard culture conditions with erythropoietin 2 U/mL, CFU-E and BFU-E colony numbers were markedly reduced in six patients and similar to adult and pediatric control values in the other two (patient nos. 2 and 8, Table 3).

Erythropoietin dose-response studies were performed with each culture by adding increasing erythropoietin concentrations in the absence or presence of GM-CSF or IL-3 (Fig 1). The response to increasing doses of erythropoietin was minimal or absent in five of the six marrows that yielded low baseline erythroid colony numbers (Table 3), but was comparable to controls in marrows from patient nos. 2 and 8.

Addition of IL-3 or GM-CSF (Fig 1) did not modify CFU-E colony numbers except for a minimal response in patient no. 6. In sharp contrast, marrow cells from five of the six patients with low baseline BFU-E colony counts (patient nos. 1, 4 to 7) showed a dramatic increase in colony numbers on addition of IL-3. Moreover, IL-3 induced in four studies (patient nos. 4 to 7) an erythropoietin dose-response pattern similar to controls. Although complete numerical correction of BFU-E colonies to control values was not observed, the growth-promoting effect of IL-3 was statistically significant at all concentrations of erythropoietin (P < 0.03). This is illustrated by Fig 2, which depicts the combined results for the six studies on patients with low baseline colony numbers. In the two patients (patient nos. 2 and 8) with baseline colony numbers that were similar to controls, BFU-E progenitors were also responsive to IL-3 (mean colony number increment of 26%), whereas CFU-E progenitors were unresponsive. Bone marrow cells from patient no. 2 that were not used for the study were frozen (-80°C) in 10% dimethylsulfoxide, thawed 7 months later, and restudied. Colony numbers and percent increment with IL-3 were comparable to the original study. Addition to erythropoietin-containing cultures of GM-CSF alone (Figs 1 and 2) or in combination with IL-3 (data not shown) did not provide any growth advantage for BFU-E colonies compared to the erythropoietin-IL-3 combination. The response to IL-3 in these studies was not related to the previous clinical response to prednisone (Table 1).

In the converse experiments, cultures with a fixed dose of erythropoietin (2 U/mL) were exposed to graded concentrations of IL-3 (dilutions of 1:1,250, 1:2,500, 1:5,000, and 1:10,000). Compared with the value in each panel in Fig 1 showing colony growth with erythropoietin 2 U/mL and IL-3 1:2,500 (8 x 10^-4), the other three IL-3 dilutions in each study did not vary more than ±20%. Thus, a concentration-dependent change in colony numbers with these dilutions of IL-3 was not observed.

IL-3 also induced impressive morphologic changes in BFU-E colony size in the cultures that were responsive to it. In the absence of IL-3, BFU-E colonies consisted of three to six small clusters of 10 to 40 cells each. Large subcolonies of over 500 cells that were frequently observed in control marrow cultures were rarely seen. On addition of IL-3, BFU-E colonies increased to six to 15 clusters, many of which consisted of 50 to 500 cells and in some cases of giant subcolonies of over 1,000 cells. BFU-E subcolonies also appeared more "cohesive" to each other in the presence of IL-3.

Baseline CFU-GM colony numbers were lower in patients' marrows compared to controls (mean ± SD = 50 ± 15 to 124 ± 36, respectively). However, both IL-3 and GM-CSF when added singly exerted a strong growth-promoting effect on CFU-GM colony proliferation and raised patients' and control colony numbers four- to five-fold. No growth advantage was seen when both agents were combined.

**DISCUSSION**

Research on hematopoietic colony-stimulating factors has recently been intensified by the development of recombinant biotechnology. As a result, the availability of large quantities of highly purified factors has opened new perspectives in the understanding and the therapeutic management of congenital and acquired bone marrow disorders.23-26

Both recombinant growth factors, IL-3 and GM-CSF, have been shown to be powerful stimulators of hematopoiesis. IL-3 is able to support the growth of multilineage erythroid, myeloid, and megakaryocytic colonies, as well as that of mast cells.23,27-33 GM-CSF, on the other hand, has a somewhat more specific activity on granulocyte and mono-
cyte-macrophage progenitor proliferation and differentiation, but is also capable of enhancing erythropoiesis and megakaryopoiesis. With in vivo studies, GM-CSF was also shown to induce a marked reticulocytosis in a pancytopenic rhesus monkey but not in humans with the acquired immunodeficiency syndrome (AIDS). IL-3 was able to raise the red cell count and hematocrit in a strain of congenitally anemic mice as well as in primates.

In this study, we examined the defective marrow erythropoiesis that characterizes Diamond-Blackfan anemia by measuring the in vitro response to the two growth factors. The initial studies identified two categories of patients based on erythropoietin-induced CFU-E and BFU-E colony proliferation, six of eight patients with decreased growth, and two others with colonies more comparable to control values. Differences in colony growth between patients have been noted before as have differences in response to corticosteroids in vitro and clinically. Thus, the clinical phenotype of Diamond-Blackfan anemia appears to be a heterogeneous disorder, although Lipton et al. have raised the possibility that the differences in erythroid colony numbers may be accounted for by the age of the patient at the time of the study. In their studies, bone marrow samples from very young patients yielded normal or only slightly reduced numbers of erythroid colonies, whereas marrow from older patients appeared severely deficient in erythroid progenitors, possibly because of a transfusion-mediated erythropoietic suppression. In keeping with their findings, the two patients in our study with normal numbers of erythroid colonies were the youngest in the series; however, three patients (patient nos. 1, 4, and 6, Table I), tested at or shortly after diagnosis, yielded very low numbers of CFU-E and BFU-E colonies, as did four other newly diagnosed patients not included in this report.

The question arises whether the differences in colony growth in our patients could be explained by the degree of...
transfusional iron overload, an effect of the chelating drug
deferoxamine, or both. Careful examination of the data
failed to identify a relationship between colony numbers and
body iron burden, dosage of deferoxamine, age of initiation
of chelation, and compliance. Moreover, three of the patients
were studied several years previously (Table 3) at a time
when the transfusional and deferoxamine history was much
shorter, and the colony numbers were comparable to those in
the current study.

Thus, differences in erythroid progenitor proliferation in
bone marrow cultures may reflect a true heterogeneity in the
pathogenesis of the defective erythropoiesis. If this hypothe-
sis is correct, the deficiency of primitive BFU-E erythroid
progenitors seen in many patients likely results from the
inability of their immediate precursor, termed CFU-GEMM,
to initiate erythroid differentiation. In patients with normal
numbers of erythroid colonies, the defect probably occurs
beyond the more differentiated CFU-E stage and prevents
terminal erythroid maturation.18

Whichever mechanism is implicated, our results indicated
that BFU-E erythroid colony proliferation was induced by
IL-3. These data confirm the current opinion that IL-3
promotes erythroid lineage proliferation more efficiently
than GM-CSF.19,21,42 The erythropoietic action of IL-3 was
characterized by an increase in the number and size of
BFU-E colonies with no observable effect on the more
mature CFU-E colonies, similar to results observed by others
in cultures of normal fractionated marrow.44 At optimal IL-3
dilutions in our studies, the increase in BFU-E colonies was
minimal in the controls (15%), moderate (26%) in the two
patients with baseline colony counts comparable to controls,
and marked (183%) in those with low baseline colony
numbers. It should be noted that the shape of the erythropoi-
etin dose-response curves varied from patient to patient
and the threshold for a response to IL-3 varied from 1 to 4
U/mL of erythropoietin. These findings further underscore
the biological diversity of this disorder.

The ability of IL-3 to restore an erythropoietin dose-
response pattern in four of six patients was remarkable.
Erythropoietin insensitivity of erythroid progenitors was
recognized previously in Diamond-Blackfan anemia and in
some cases was partially corrected by the addition of cortico-
steroids to the culture media.13,15,16 One can postulate that
IL-3, as well as corticosteroids, are able to enhance the
response to erythropoietin of erythroid progenitors, possibly
by an “up-regulating” effect on erythropoietin receptors.
However, overlapping activities of IL-3 and corticosteroids in
this regard probably result from different mechanisms since
corticosteroids act at the level of both BFU-E and CFU-E
progenitors,16,17 whereas IL-3 specifically enhances the BFU-
E response to erythropoietin. Moreover, no correlation was
found in our studies between the clinical response to predni-
sone and the IL-3 response in vitro.

Our results indicate that in some patients, the defective
erythroid progenitors in Diamond-Blackfan anemia are able
to proliferate and differentiate better in an IL-3 enriched
environment. Although correction of the number of BFU-E
colonies was partial in most marrows studied, in two cases
(patient nos. 4 and 6, Fig 1), the numbers obtained at high
concentrations of erythropoietin and in the presence of IL-3
approached the lower limits of control values. Considering
that patients with Diamond-Blackfan anemia usually have
high serum levels of erythropoietin,3 it is not unreasonable to
hope that the provision of exogenous IL-3 would result in an
optimal interaction of both erythropoietic growth factors and
achieve a clinically valuable outcome. Since colony numbers
and peripheral blood counts do not always correlate (for
example, patient nos. 2 and 8), the predictive value of cell
cultures for clinical outcome in response to IL-3 therapy may
be limited. Nevertheless, provided that preliminary clinical
studies establish safety, an in vivo trial of IL-3 in Diamond-
Blackfan anemia would constitute a logical and timely
continuation of this report.

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Diamond-Blackfan anemia: promotion of marrow erythropoiesis in vitro by recombinant interleukin-3

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