In Vitro Growth and Regulation of Bone Marrow Enriched CD34+ Hematopoietic Progenitors in Diamond-Blackfan Anemia


Diamond-Blackfan anemia (DBA) is a congenital red blood cell aplasia. No clear explanation has been given of its defective erythropoiesis, although different hormonal or cellular inhibitory factors have been proposed. To clarify the nature of this defect we studied the effect of several human recombinant growth factors on an enriched CD34+ population obtained from the bone marrow of 10 DBA patients. We observed a defect underlying the early erythroid progenitors, which were unresponsive to several growth factors (erythropoietin, interleukin-3 [IL-3], IL-6, granulocyte-macrophage colony-stimulating factor [GM-CSF], erythropoietin potentiating activity), either alone or in association. The production of cytokines was not impaired, and high levels of IL-3 and GM-CSF were found in phytohemagglutinin-leukocyte-conditioned medium (PHA-LCM) when tested with a sensitive biologic assay on the M-07E cell line. Hematopoietic stem cells in DBA patients may be induced to differentiate to the granulocyte macrokaryocyte, but not the erythroid compartment, as shown after CD34+ cell preincubation with IL-3. Addition of the stem cell factor to IL-3 and erythropoietin induces a dramatic in vitro increase in both the number and the size of BFU-E, which also display a normal morphologic terminal differentiation.

© 1991 by The American Society of Hematology.

MATERIALS AND METHODS

Patients and methods. Bone marrow samples were obtained from 10 DBA patients and 12 normal controls of the same age after

From the Institute of Histology and General Embryology, “Giorgio Prodi” Interdepartmental Center for Cancer Research, and the Department of Pediatrics III, University of Bologna; the Department of Biomedical Sciences and Human Oncology and the Department of Pediatrics, University of Turin, Italy.

Supported in part by an Italian Ministry of University and Scientific and Technological Research Grant, and A.I.R.C. grants to Dr L. Pegoraro and G. P. B.

Address reprint requests to Gian Paolo Bagnara, MD, Institute of Histology and General Embryology, University of Bologna, Via Belmerico 8, Bologna, Italy.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.

0006-4971/91/17809-0032$3.00/0

BLOOD, Vol 78, No 9 (November 1, 1991): pp 2203-2210

2203
informed consent. Bone marrow aspirates were taken from controls for suspected hematologic disease not confirmed by subsequent analysis.

Peripheral blood samples (15 mL) were collected from these patients and 10 normal donors to prepare phytohemagglutinin-leukocyte-conditioned medium (PHA-LCM).

The patients were four boys and six girls, ages 2 months to 16 years (mean 7 ± 5 years), all diagnosed within the first 5 months of life (except for one at 2 years) on the basis of anemia with low reticulocyte count and marrow erythroid hypoplasia (erythroid nucleated cells 0.3% to 16.8%, mean 5.1% ± 4.9%). In case 8, maturation had been arrested at the proerythroblast level. Clinical and hematologic data at diagnosis and the time of the study are summarized in Table 1. All patients were studied at least 6 months after suspension of steroid therapy or before its commencement (cases 5 and 8). Serum Epo levels were higher than expected for the current hemoglobin (Hb) level (mean Epo 448 ± 254 mU/mL, mean Hb 9.4 ± 0.77 g/dL).

Three patients presented congenital abnormalities (anophthalmus, ventricular septal defect, ventricular septal defect, plus pulmonary valvular stenosis).

Recombinant growth factors. Recombinant human (rh) GM-CSF, rhIL-6, and rhIL-3 were kindly supplied by Behring (Behring, Marburg, Germany) and stored at -70°C for no more than 3 months. Ficoll-Hypaque (1077 sd; Pharmacia, Uppsala, Sweden) and centrifuged at 1,500 rpm for 30 minutes. Light-density mononuclear cells (LD-MC) were collected and washed twice in IMDM supplemented with 10% of fetal calf serum (FCS). Mononuclear adherent cells were then removed by two steps of incubation (1 hour) in plastic flasks at 37°C in a humidified atmosphere of 5% CO2 in air. Subsequently, T-lymphocyte-depleted MNAC were obtained by two steps of rosetting with neuraminidase-treated sheep RBCs. After removal of T cells, a positive reaction to the OK T11 (CD2) monoclonal antibody (MoAb) was constantly less than 1%. In cases 3, 9, and 10, the lymphocytes were negatively removed with immunomagnetic beads coated with antimmunoglobulin IgG (M-450 Dynabeads; Dynal, Oslo, Norway) after incubation at 4°C with the following antibodies: CD2, CD19, CD22, CD14, CD51, and CD57.

Positive selection of CD34+ cells in bone marrow samples. Bone marrow non-T MNAC, 5 × 106, were treated with 5 μL of My10 MoAb (CD34+ Tycogenetics, Milan, Italy) for 1 hour at 4°C, in a final volume of 150 to 200 μL of phosphate-buffered saline (PBS) + 1% FCS, under continuous shaking.

After two washings to eliminate the excess MoAb, cells were treated with immunomagnetic beads coated with antimmunoglobulin IgG for 30 minutes in ice. A ratio of 3 to 4 beads per target cell was found to provide the best recovery. CD34+ cells were then collected by a magnet (MPC 1, Dynabeads; Dynal) and resuspended in IMDM + 1% FCS, under continuous shaking.

For personal use only.

Table 1. Hematologic and Clinical Data

<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>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G.M</td>
<td>L.D</td>
<td>T.M</td>
<td>C.E</td>
<td>C.A</td>
<td>M.S</td>
<td>T.L</td>
<td>C.S</td>
<td>P.A</td>
<td>S.P</td>
</tr>
<tr>
<td>At presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Age (mo)</td>
<td>2</td>
<td>5</td>
<td>24</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1.5</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Congenital anomalies*</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Hb g/dL</td>
<td>3.5</td>
<td>6.8</td>
<td>3</td>
<td>3.2</td>
<td>2.4</td>
<td>2.3</td>
<td>6.6</td>
<td>4.6</td>
<td>7.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Reticulocytes %</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>WBC × 109/L</td>
<td>6.8</td>
<td>13</td>
<td>3.5</td>
<td>9.9</td>
<td>9.9</td>
<td>10.2</td>
<td>9.7</td>
<td>11.4</td>
<td>7.2</td>
<td>8.5</td>
</tr>
<tr>
<td>Platelets × 109/L</td>
<td>250</td>
<td>200</td>
<td>200</td>
<td>150</td>
<td>450</td>
<td>200</td>
<td>440</td>
<td>492</td>
<td>330</td>
<td>320</td>
</tr>
<tr>
<td>Bone marrow % erythroid nucleated cells</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>1.5</td>
<td>0.4</td>
<td>16.5</td>
<td>1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Epo mU/mL</td>
<td>380</td>
<td>400</td>
<td>350</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At the moment of study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>16</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>2 (mo)</td>
<td>5</td>
<td>11</td>
<td>7 (mo)</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Hb g/dL</td>
<td>9.5</td>
<td>9</td>
<td>10.5</td>
<td>10</td>
<td>9.8</td>
<td>8.3</td>
<td>9.1</td>
<td>6.8</td>
<td>10.3</td>
<td>8.3</td>
</tr>
<tr>
<td>Reticulocytes %</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.05</td>
<td>0.05</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>WBC × 109/L</td>
<td>4.5</td>
<td>5.8</td>
<td>6.7</td>
<td>8.2</td>
<td>7.2</td>
<td>7.3</td>
<td>4.0</td>
<td>12.8</td>
<td>4.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Platelets × 109/L</td>
<td>220</td>
<td>230</td>
<td>320</td>
<td>300</td>
<td>250</td>
<td>150</td>
<td>231</td>
<td>401</td>
<td>36</td>
<td>384</td>
</tr>
<tr>
<td>Regular transfusion therapy</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Steroid response</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Epo mU/mL</td>
<td>600</td>
<td>193</td>
<td>822</td>
<td>349</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>320</td>
<td>169</td>
<td>686</td>
</tr>
</tbody>
</table>

*Congenital anomalies: ventricular septal defect patient 2, ventricular septal defect + pulmonary stenosis patient 8, anophthalmus patient 7.

+BM % erythroid cells: patient 8 maturative arrest at the proerythroblast level.
CD4, CD2, CD16, and CD19 (Ortho Diagnostic System, Raritan, NJ).

Cell culture. The colony assay for BFU-E was performed according to Iscove et al.2 Bone marrow CD34+ cells, 106, resuspended in 0.1 mL of PBS + 1% detoxified bovine serum albumin (BSA; Fraction V Chon; Sigma, St Louis, MO) were plated in a 1-mL mixture of IMDM containing 30% FCS, 2 × 10-4 mol/L hemin, 5 × 10-3 mol/L β-mercaptoethanol, 1% BSA, and 0.9% methylcellulose. The cells were stimulated in our optimal standard conditions, with rhEpo 2 U and rhIL-3 100 U or with various combinations of recombimant growth factors, as specified below.

The assay for the 14-day CFU-GM was performed as previously described.14 CD34+ bone marrow cells, 106, were plated in 35-mm Petri dishes in 1 mL of IMDM containing 20% heat-inactivated FCS, 0.3% noble agar, and 200 U rhGM-CSF + 100 U of rhIL-3 as the standard source of colony-stimulating activity.

For the megakaryocyte colony-forming units (CFU-MK) assay, plasma clot assay was performed according to McLeod et al.12 and Vainchenk et al.15 CD34+ cells, 106, were seeded in IMDM, supplemented with 20 µg L-Asparagine (Sigma) 3.4 µg CaCl2, 10% BSA, 10% of a preselected batch of heat-inactivated pooled human AB serum, and 200 U rhGM-CSF. After 12 days of incubation, the plasma clot was fixed in situ with methanol-acetone 1:3 for 20 minutes, washed with PBS and double-distilled water, and then air-dried. Fixed plates were stored at −20°C until immunofluorescence staining was performed. CFU-MK colonies were scored as aggregates of 3 to 100 cells intensively fluorescent to J15 (CD41W) MoAb directed against the IIb-IIIa glycoprotein complex. Binding was shown by fluorescent goat antimouse IgG (Ortho).

To clarify whether different combinations of recombinant factors influence bone marrow progenitors, and especially BFU-E growth of Epo 2 to 6 U/mL alone or in combination with: IL-6 (1 to 100 U/mL), GM-CSF (200 U/mL), IL-3 (100 U/mL), EPO (1:100), IL-3 (100 U) plus GM-CSF (200 U), IL-3 (100 U) plus IL-6 (1 to 100 U/mL). In three patients (cases 3, 9, and 10) and in three normal controls the effect of the SCF on BFU-E growth was studied on the CD34+ positive bone marrow population. The SCF has been tested at the concentrations of Epo 2 to 6 U/mL alone or in combination with:

\[ \text{Epo} 2 \text{to} 6 \text{U/mL} \] 
\[ \text{IL-3 (100 U/mL)} \] 
\[ \text{IL-6 (1 to 100 U/mL)} \] 
\[ \text{GM-CSF (200 U/mL)} \] 
\[ \text{SCF (1:100, L:200, and 1:400 in association with Epo 2 and 4 U/mL, and IL-3 (100 U/mL).} \]

Pre-exposure experiments in liquid cultures. CD34+ cells from four patients and four controls were either seeded immediately in semisolid media or preincubated in IMDM + 10% FCS in the presence of rhIL-3 (100 U/mL). Daily (up to the fourth day) aliquots were collected for clonogenic assays. The percentage of CD34+ cells was determined by immunofluorescence. BFU-E, CFU-GM, and CFU-MK growth is expressed as the number of colonies by 106 CD34+ cells.

Preparation of PHA-LCM. LD-MC from the peripheral blood of the 10 DBA patients and 10 normal donors were seeded in IMDM, supplemented with 10% FCS and 1% phytomenadionin (PHA) at a concentration of 106 cells/mL, and incubated for 72 hours. The conditioned medium was then harvested, centrifuged for 10 minutes at 2,000 rpm, filtered, and stored at −70°C for no more than 3 months, before use on the M-07E cell line.

Anti–GM-CSF and anti–IL-3. Murine MoAb anti–GM-CSF and anti–IL-3 were purchased fromGenzyme Co (Cambridge, MA). A fixed dilution of 1:100 (PBS + 1% FCS) was tested on 0.005% to 10% vol/vol concentrations of both normal and DBA patients’ PHA-LCM.

Biologic assay for the production of GM-CSF and IL-3. The GM-CSF and IL-3-dependent M-07E cell line17 was kindly provided by Dr L. Pegoraro (University of Turin, Italy). PHA-LCM was used as a source of growth factors at a final concentration of 1% for 96 hours. Exponentially growing M-07E cells were washed twice and seeded at 1 × 106 cells/mL in IMDM with 5% FCS. After 24 hours of IL-3 deprivation, 5 × 106 cells were washed twice and seeded in 200-µL microwells in IMDM with 5% FCS in the presence of increasing concentrations of PHA-LCM (from 0.005% to 10% vol/vol). After 28 hours, triplicate wells were pulse-labeled with [3H]-Tdr (1 µCi/well) for 4 hours. Alcohol acid precipitated radioactivity was determined by liquid scintillation counting.

The concentration of IL-3 and GM-CSF in PHA-LCM was determined by comparing the dose-response curves obtained with serial dilutions of rhIL-3 and rhGM-CSF and of PHA-LCM. GM-CSF and IL-3 levels were determined by subtraction of the number of counts per minute (cpm) obtained in the presence of the anti–IL-3 or anti–GM-CSF MoAb from the total cpm amount.

Epo measurement. Serum Epo levels were determined by radioimmunoassay using radiolabeled hormone and anti-Epo antibodies (Ingenta; Sorin, Saluggia, Italy).

Statistical analysis. The results were expressed as means ± 1 standard error of mean (SEM) of the data from two or more experiments performed in duplicate. Statistical analysis was performed using the two-tailed Student’s t-test, linear regression, and correlation tests.

RESULTS

In vitro growth of enriched (CD34+) bone marrow cells. The results of the in vitro growth of 14th day CFU-GM, CFU-MK, and BFU-E in our standard culture conditions are summarized in Table 2.

Our assay did not assess CFU-E growth. Culture of CD34+ cells does not give rise to CFU-E colonies at day 7 because they are earlier progenitors, and especially the 14th day BFU-E are detectable.

The number of CFU-GM and CFU-MK colonies derived from DBA marrows was similar to that from normal controls (P > .05). In contrast, a dramatic decrease in the erythroid progenitors was observed in 9 of 10 DBA patients (P < .01).

Production of IL-3 and GM-CSF from PHA-LCM of DBA patients. We investigated whether production of the cytokines involved in erythroid differentiation accounts for impaired erythropoiesis in DBA patients. The production of IL-3 and GM-CSF by DBA patient and normal control

<table>
<thead>
<tr>
<th>Case No.</th>
<th>DBA</th>
<th>BFU-GM</th>
<th>BFU-MK</th>
<th>BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>22</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>35</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>32</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>23</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>17</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>18</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>16</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SEM: 18.9 ± 2.2 20.7 ± 2.4 4 ± 2.3

Controls (mean of 12 cases ± SEM): 29 ± 6.5 23.2 ± 16 62 ± 14

From www.bloodjournal.org by guest on November 15, 2017. For personal use only.
peripheral blood mononuclear cells was tested on the M-07E cell line.

Addition of 1:100 vol/vol anti-GM-CSF and anti-IL-3 completely abrogated the stimulatory activity of 10 pg/mL IL-3 and 2 to 3 pg/mL GM-CSF, respectively (data not shown). However, in the experiments performed on PHA-LCM, incomplete neutralization with anti-GM-CSF and anti-IL-3 MoAbs was observed at the higher PHA-LCM concentrations (1% and 10%) (Fig 1). This finding may be partially explained by the presence of other cytokines (ie, IL-2, IL-4, and IL-9) to which the M-07E line is sensitive, although not dependent on them.29

The amount of both IL-3 and GM-CSF was significantly higher \((P < .01)\) in DBA patients: 384 ± 27 pg IL-3 and 302 ± 12 pg GM-CSF versus 206 ± 15 pg and 230 ± 9.2 pg in normal controls. The difference in IL-3 production was statistically significant at the final PHA-LCM concentration of 10% \((P = .034)\), whereas that in GM-CSF levels was only significant at 0.5% \((P = .001)\) and 0.1% \((P = .003)\), indicating that IL-3 production is more pronounced than that of GM-CSF in DBA.

Pre-exposure to IL-3. Exposure to IL-3 increases the number of all the hematopoietic progenitors and induces their differentiation.30 Therefore, we investigated the effect of a 12- to 96-hour exposure of bone marrow CD34+ cells to 100 U of rhuIL-3. After 12, 24, 48, 72, and 96 hours, CD34+ cells were harvested and seeded in methylcellulose medium for BFU-E growth. Data represent the means ± SEM of the number of colonies observed in four separate experiments. Each point was performed in duplicate.

Effect of cytokines on the in vitro growth of BFU-E. The effect of different combinations of recombinant factors on the growth of control and DBA BFU-E is illustrated in Table 3. Hemoglobinized erythroid colonies were never detected in the absence of Epo. In CD34+ enriched normal bone marrow cultures, these combinations increased the number of BFU-E compared with Epo alone, peak stimulation being achieved by Epo (2 U) + IL-3 (100 U). DBA BFU-E were never increased.

The response of the erythroid progenitors to different dilutions of SCF in three DBA cases and in normal controls is illustrated in Table 4. Addition of even the lowest SCF dilution to Epo and IL-3 induced a dramatic increase in the number of both normal and DBA BFU-E. This finding was the same when Epo was increased from 2 to 4 U/mL (data not shown). There was a striking increase of the size of each aggregate in a single BFU-E (from a mean of 30 cells to more than 200 cells) (Fig 4).

DISCUSSION

Several reasons have been proposed for the erythropoietic inhibition observed in DBA: a humoral mechanism involving autoimmunity or the presence of inhibiting factors,31,32 a cellular mechanism mediated by T lymphocytes, NK cells, or adherent cells,9,12 impaired cytokine production
REGULATION OF HEMATOPOIESIS IN DBA

A decrease in bone marrow BFU-E and CFU-E number in DBA has been observed.  

In contrast, attempts to enhance erythroid progenitor growth and differentiation have given conflicting results. The growth defect can be partially corrected in vitro by addition of high doses of Epo or IL-3 or steroids.  

Interestingly, the impairment of in vivo erythropoiesis seems to be more dramatic in DBA patients who do not respond to steroid therapy.  

An increased number of reticulocytes and a decreased blood requirement in some transfusion-dependent and steroid-unresponsive DBA patients have been recently reported following the administration of GM-CSF and IL-3.  

All these findings suggest a possibility of improving erythroid growth. The results obtained have always been scanty and transient. Although this could be due to differences in the genetic defect, patient’s age and clinical history, it must be underlined that the bone marrow progenitors have not always been concentrated and purified from accessory cells, and recombinant growth factors were not used by some workers.  

In our experiments, only CD34+ positively selected progenitors, virtually free from accessory cells, were plated in the presence of well-defined recombinant growth factors. We also investigated the ability of patient’s mononuclear cells to produce GM-CSF and IL-3, which are well-known regulators of erythropoiesis.  

Our observation that addition of various recombinant cytokines to a CD34+ positively selected lymphocyte and monocyte-free marrow population always fails to support the growth of BFU-E in DBA challenges the supposition of a direct lymphocyte or monocyte involvement in the BFU-E growth defect.  

The inhibition of erythropoiesis reported by some investigators in cocultures with T lymphocytes from DBA patients may be ascribable to an immunologic phenomenon related to HLA antigens in subjects regularly transfused.  

After pre-exposure to IL-3, hematopoietic progenitors were induced to differentiate to the granulocyte and the megakaryocyte, but not the erythropoietic lineage. This finding indicates that the low number of BFU-E is not due to a diminished population of multipotent stem cells, but to a defect in the passage from the CFU-GEMM to BFU-E as shown by the lack of progenitor response to the in vitro stimulating activity of Epo and IL-3, IL-6, GM-CSF, EPA, alone or in association.  

Moreover, impaired production of these cytokines in DBA is denied in our experiments. In a highly sensitive biologic assay, PHA-LCM produced increased amounts of IL-3 and, to a lesser extent, GM-CSF. However, if we take into account the central role played by IL-3 and by GM-CSF in normal bone marrow BFU-E growth and differentiation, it is conceivable that these increases, along

![Graph](image)

Fig 3. CFU-GM and CFU-MK growth in agar and plasma clot media. CD34+ cells from four DBA patients and four normal controls were pre-exposed to IL-3 for 96 hours. Data are the means ± SEM of four separate experiments. Each point was performed in duplicate. ( ), CFU-GM DBA; ( ), CFU-GM controls; ( ), CFU-MK DBA; ( ), CFU-MK controls.

or function, or an intrinsic defect within the hematopoietic stem cells.  

A decrease in bone marrow BFU-E and CFU-E number in DBA has been observed.  

In contrast, attempts to enhance erythroid progenitor growth and differentiation have given conflicting results. The growth defect can be partially corrected in vitro by addition of high doses of Epo or IL-3 or steroids.  

Interestingly, the impairment of in vivo erythropoiesis seems to be more dramatic in DBA patients who do not respond to steroid therapy.  

An increased number of reticulocytes and a decreased blood requirement in some transfusion-dependent and steroid-unresponsive DBA patients have been recently reported following the administration of GM-CSF and IL-3.  

All these findings suggest a possibility of improving erythroid growth. The results obtained have always been scanty and transient. Although this could be due to differences in the genetic defect, patient’s age and clinical history, it must be underlined that the bone marrow progenitors have not always been concentrated and purified from accessory cells, and recombinant growth factors were not used by some workers.  

In our experiments, only CD34+ positively selected progenitors, virtually free from accessory cells, were plated in the presence of well-defined recombinant growth factors. We also investigated the ability of patient’s mononuclear cells to produce GM-CSF and IL-3, which are well-known regulators of erythropoiesis.  

Our observation that addition of various recombinant cytokines to a CD34+ positively selected lymphocyte and monocyte-free marrow population always fails to support the growth of BFU-E in DBA challenges the supposition of a direct lymphocyte or monocyte involvement in the BFU-E growth defect.  

The inhibition of erythropoiesis reported by some investigators in cocultures with T lymphocytes from DBA patients may be ascribable to an immunologic phenomenon related to HLA antigens in subjects regularly transfused.  

After pre-exposure to IL-3, hematopoietic progenitors were induced to differentiate to the granulocyte and the megakaryocyte, but not the erythropoietic lineage. This finding indicates that the low number of BFU-E is not due to a diminished population of multipotent stem cells, but to a defect in the passage from the CFU-GEMM to BFU-E as shown by the lack of progenitor response to the in vitro stimulating activity of Epo and IL-3, IL-6, GM-CSF, EPA, alone or in association.  

Moreover, impaired production of these cytokines in DBA is denied in our experiments. In a highly sensitive biologic assay, PHA-LCM produced increased amounts of IL-3 and, to a lesser extent, GM-CSF. However, if we take into account the central role played by IL-3 and by GM-CSF in normal bone marrow BFU-E growth and differentiation, it is conceivable that these increases, along

![Table](image)

Table 3. Effect of Different Combinations of Growth Factors on the In Vitro Growth of Erythroid Progenitors (BFU-E) From Enriched Bone Marrow Samples (106 CD34+ cells/plate) of 10 DBA Patients and 12 Normal Controls

<table>
<thead>
<tr>
<th>Case</th>
<th>Controls</th>
<th>DBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Epo 2 U</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>Epo 4 U</td>
<td>30 ± 13</td>
</tr>
<tr>
<td>3</td>
<td>Epo 6 U</td>
<td>32 ± 13</td>
</tr>
<tr>
<td>4</td>
<td>Epo 2 U + IL-3 100 U</td>
<td>62 ± 14</td>
</tr>
<tr>
<td>5</td>
<td>Epo 2 U + IL-3 100 U + IL-6 1 U</td>
<td>45 ± 13</td>
</tr>
<tr>
<td>6</td>
<td>Epo 2 U + IL-3 100 U + IL-6 10 U</td>
<td>46 ± 14</td>
</tr>
<tr>
<td>7</td>
<td>Epo 2 U + IL-3 100 U + IL-6 100 U</td>
<td>60 ± 15</td>
</tr>
<tr>
<td>8</td>
<td>Epo 2 U + IL-6 1 U</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>9</td>
<td>Epo 2 U + IL-6 10 U</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>10</td>
<td>Epo 2 U + IL-6 100 U</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>11</td>
<td>Epo 2 U + GM-CSF 200 U</td>
<td>45 ± 13</td>
</tr>
<tr>
<td>12</td>
<td>Epo 2 U + GM-CSF 200 U + IL-3 100 U</td>
<td>60 ± 14</td>
</tr>
<tr>
<td>13</td>
<td>Epo 2 U + EPA L:100</td>
<td>55 ± 14</td>
</tr>
</tbody>
</table>

Data are expressed as means ± 1 SEM. Each point was performed in triplicate.
with the well-documented elevation of serum Epo in DBA, are an attempt to compensate for erythropoietic failure.

The in vivo cytokine network is still poorly defined and other factors may be involved in stem cell expansion and erythroid differentiation. The recently described hematopoietic SCF (also called mast cell growth factor, steel locus factor, c-kit ligand) has been shown to induce an increased proliferation of hematopoietic progenitors and to display a potent synergistic activity in combination with IL-3 and other cytokines. SCF also seems to play an important role in the activation of primitive cells, while the differentiation signal is provided by the specific CSFs. Moreover, its in vivo administration reverses anemia in Sl/Sld mutant mice.

In our experiments, in vitro addition of SCF to IL-3 and Epo induced a significant increase of the number and size of both normal and DBA BFU-E. This effect was constant and striking. Stimulation of DBA erythroid progenitors was observed at the SCF concentration range that provides optimal growth enhancement on normal CD34⁺ cells and M-07E cells (S.C. Clark, unpublished data), suggesting that in DBA, SCF receptor is normally expressed on SCF-responsive CD34⁺ cells. The hypothesis that other receptor molecules, such as Epo receptors, are involved in the disease can not be ruled out. The ability of SCF to induce BFU-E proliferation and differentiation in DBA indicates a similarity between this disease and the murine complex.

Fig 4. Size of erythroid aggregates from DBA CD34⁺ bone marrow cells. (A) Cultures stimulated with Epo (2 U/mL) + IL-3 (100 U/mL); (B) cultures stimulated with Epo (2 U/mL) + IL-3 (100 U/mL) and SCF 1:200 vol/vol.
REGULATION OF HEMATOPOIESIS IN DBA

2209

hematopoietic defect caused by mutations of SI and W genes.\textsuperscript{36}

In conclusion, our findings show that SCF significantly reverses the erythropoietic impairment in DBA in vitro, suggesting a possible therapeutic utility in vivo.

Further studies are needed to see whether the inability of the bone marrow to produce or release a biologically active form of this factor is involved in the pathogenesis of DBA.

ACKNOWLEDGMENT

We thank Dr. S.C. Clark for kindly providing SCF and Dr. L. Pegoraro for helpful discussion.

REFERENCES

20. Flanagan JG, Chan DC, Leder P: Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the SI\textsuperscript{+} mutant. Cell 64:1025, 1991
21. Golde DW, Bress N, Quan SG, Lusis AJ: Production of erythropoietin-potentiating activity by a human T-lymphoblast cell line. Proc Natl Acad Sci USA 77:593, 1980
32. Marmont A, Peschle C, Sanguineti M, Cordorelli M: Response of three patients to cyclophosphamide and/or antilymphocyte globulin (ALG) and demonstration of two types of serum IgG inhibitors to erythropoiesis. Blood 45:247, 1975


In vitro growth and regulation of bone marrow enriched CD34+ hematopoietic progenitors in Diamond-Blackfan anemia

GP Bagnara, G Zauli, L Vitale, P Rosito, V Vecchi, G Paolucci, GC Avanzi, U Ramenghi, F Timeus and V Gabutti