Bone marrow cells from seven patients with preleukemia and two with erythroleukemia were cultured in vitro to characterize erythropoiesis and erythropoietin responsiveness in these disorders. The methylcellulose plate technique was used to clone erythroid progenitors, and the results were compared to data obtained from studies on five healthy volunteers. Erythroid colonies formed in cultures from all patients and controls, and a clear erythropoietin dose-response effect was observable in each case. The erythroid cloning efficiency of bone marrow cells from the preleukemia patients varied considerably and in some cases was greater than normal. The erythropoietin dose-response relationships were linear on a log-log plot over the erythropoietin concentrations tested (0.05–2.0 U/ml), and linear regression equations generated for each patient and patient group had excellent fits. The mean slopes of the regression lines for preleukemia, polycythemia vera, and normal were not statistically different, suggesting that erythroid precursors in these disorders have similar erythropoietin responsiveness in vitro. Increased cloning was observed with the addition of dexamethasone (10^{-9} M) to cultures, indicating that glucocorticoid sensitivity was retained by erythroid colony-forming cells in these disorders. These studies show that red cell precursors in preleukemia and erythroleukemia are capable of colony formation in vitro and show erythropoietin responsiveness. The findings also suggest that the marrow pool of erythroid colony-forming cells may be expanded in some cases of preleukemia and that erythroid colony formation in vitro is not correlated with effective erythropoiesis in vivo.

A CUTE MYELOGENOUS LEUKEMIA (AML) is usually associated with morphologic and functional abnormalities of erythropoiesis,\textsuperscript{1,2} and cytogenetic studies have provided evidence that many of the red blood cell precursors in AML are derived from the leukemic clone.\textsuperscript{3} Perhaps the most compelling findings were those of Blackstock and Garson,\textsuperscript{4} who showed that erythroid cells incorporating \textsuperscript{59}Fe contained the same chromosomal abnormality seen in myeloid leukemic blasts.

Preleukemia is a recognizable syndrome of hematopoietic dysfunction that precedes the classical manifestations of AML.\textsuperscript{5,8} Anemia is almost invariably present in preleukemia, and disordered erythropoiesis is prominent. The red cell precursors in the marrow are often megaloblastic, and morphologic evidence of abnormal erythroid maturation may be dramatic. Ringed sideroblasts are also a frequent finding.\textsuperscript{1,7} Erythrokinetic studies in preleukemia have confirmed the clinical evidence of ineffective erythropoiesis.\textsuperscript{9} Although strict proof is lacking, these morphologic, cytogenetic, and kinetic studies suggest that erythroid involvement in preleukemia occurs as part of the neoplastic transformation at a pluripotent stem cell level.\textsuperscript{10}
The present investigation was undertaken to characterize erythropoiesis in vitro in preleukemia and erythroleukemia and to determine the erythropoietin responsiveness of the erythroid precursors in these disorders. Studies of hormonal responsiveness in vitro were extended to include glucocorticoid-potentiating effects on erythroid precursor proliferation. Erythropoiesis in preleukemia and erythroleukemia was compared to that of normal subjects and previously studied patients with polycythemia rubra vera.11

MATERIALS AND METHODS

Cell culture. Bone marrow was aspirated from the posterior iliac crest of appropriately informed healthy adult volunteers and patients. The heparinized cell suspension was passed through progressively smaller bore needles until it easily passed through a 25-gauge needle. Nucleated cells were isolated by centrifugation at 500 g in Wintrobe tubes and washed twice in tissue culture medium. The methylcellulose technique was used to grow erythroid colonies in vitro.12,13 Nucleated cells (2 × 10⁶) were plated in 0.8% methylcellulose with a medium containing 30% fetal calf serum, penicillin, streptomycin, and 10⁻⁴ M α-thioglycerol. Duplicate plates were incubated for 7-8 days in a humidified atmosphere of 7.5% CO₂ in air at 37°C, and erythroid colonies composed of eight or more hemoglobinized cells were counted using an inverted microscope. Random colonies were transferred to glass slides and stained with benzidine to confirm their erythroid nature.

Human urinary erythropoietin (Ep) (obtained from the National Heart, Lung and Blood Institute) (73 U/mg protein) was added to cultures in concentrations of 0.05-2 U/ml. Fetal calf

Table 1. Clinical Characteristics of Patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex/Age (yr)</th>
<th>PCV (%)</th>
<th>WBC (/μl)</th>
<th>Platelets (/μl)</th>
<th>M:E*</th>
<th>Cellularity</th>
<th>Bone Marrow RBC Morphology</th>
<th>Cytogenetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preleukemic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F/61</td>
<td>20</td>
<td>2700</td>
<td>43,000</td>
<td>1.5:1</td>
<td>Megaloblastic†</td>
<td>46,xx</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M/77</td>
<td>16</td>
<td>8000</td>
<td>30,000</td>
<td>4.5:1</td>
<td>Megaloblastic</td>
<td>47,xx (+8)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M/77</td>
<td>25</td>
<td>3300</td>
<td>60,000</td>
<td>1.0:1</td>
<td>Megaloblastic with ring sideroblasts</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>F/25</td>
<td>18</td>
<td>8600</td>
<td>125,000</td>
<td>3.0:1</td>
<td>Megaloblastic</td>
<td>45,xx (−C)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M/57</td>
<td>20</td>
<td>3500</td>
<td>35,000</td>
<td>3.0:1</td>
<td>Dysplastic</td>
<td>46,xy</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F/74</td>
<td>33</td>
<td>5000</td>
<td>150,000</td>
<td>1.5:1</td>
<td>Megaloblastic with ring sideroblasts</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M/75</td>
<td>21</td>
<td>4000</td>
<td>50,000</td>
<td>2.5:1</td>
<td>Megaloblastic, dysplastic</td>
<td>46,xy</td>
<td></td>
</tr>
<tr>
<td>Erythro- leukemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M/64</td>
<td>22</td>
<td>5800</td>
<td>50,000</td>
<td>1.0:1</td>
<td>Megaloblastic, dysplastic</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F/69</td>
<td>23</td>
<td>1900</td>
<td>40,000</td>
<td>1.0:1</td>
<td>PAS+ vacuoles</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Polycythemia vera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-14</td>
<td>M,F/37-75</td>
<td>43-58</td>
<td>N†</td>
<td>N†</td>
<td>3.0:1-</td>
<td>1.5:1</td>
<td>N† N†</td>
<td>—</td>
</tr>
<tr>
<td>Normals</td>
<td>M,F/30-60</td>
<td>38-45</td>
<td>N</td>
<td>N</td>
<td>3.0:1</td>
<td>N N</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

† indicates increased; N, normal.
†Auer rods present in myeloblasts.
‡Numerous chromosomal abnormalities.
ERYTHROPOIESIS IN PRELEUKEMIA

13

serum containing approximately 0.035-0.05 U/ml Ep (bioassay) was used in all experiments and contributed about 0.015 U of Ep to each plate. Dexamethasone (Sigma Chemical, St. Louis, Mo.) was dissolved in ethanol, diluted in medium, and added to selected cultures. Appropriate culture studies were performed to control for a possible effect of the steroid diluent.

Regression equations were generated using logarithmic Ep dose-response data for each patient and mean regression lines calculated using a standard computer program. The slopes were calculated and compared to control.

Patients. We studied seven patients with preleukemia, two with erythroleukemia, and five normal healthy volunteers (Table 1). Five previously reported patients with polycythemia vera were included for comparison.

The seven preleukemia patients presented with typical manifestations of the syndrome and had peripheral blood and bone marrow abnormalities consistent with this condition as described in the literature. All preleukemic patients were anemic with low corrected reticulocyte counts, and all but one patient (No. 6) required frequent blood transfusions. Bone marrow aspirates and biopsies at the time of study were judged to be hypercellular in six patients, and there was dysplastic and megaloblastic erythroid maturation in the entire group. Abnormalities in granulocyte differentiation at the time of study were insufficient to diagnose overt myeloid leukemia. Patient No. 2 had a trisomy 8 cytogenetic abnormality in all 100 bone marrow metaphases examined from three marrow aspirates taken at 2-mo intervals. Patient No. 4 had a C-group monosomy in all 35 marrow metaphases examined in one marrow aspirate.

Patients 1, 2, 4, 5, and 7 developed typical AML subsequent to study. Patient No. 3 died of congestive heart failure 2 mo after the bone marrow study while he was still in the preleukemic stage. Patient No. 6 continues in the preleukemic state 6 mo after study.

Two patients with the clinical and morphologic criteria of erythroleukemia (patients 8 and 9) were studied. Bone marrow aspirates showed marked erythroid hyperplasia with prominent megaloblastic and dysplastic abnormalities, numerous ring sideroblasts, and multinucleated red cell precursors. Globular periodic acid-Schiff (PAS)-reactive material was found in many of the early erythroid precursors. At the time of study 15'-20% of the marrow cells were myeloblasts. Both patients refused chemotherapy and developed AML and died of sepsis within 3 mo of study.

RESULTS

Bone marrow cells from the five normal subjects formed erythroid colonies in vitro in response to Ep stimulation. As previously described, the number of colonies formed was a direct function of Ep concentration. A mean of 69 ± 21 colonies formed from 2 × 10^5 marrow cells at an Ep concentration of 1 U/ml. Erythroid colonies also formed in methylcellulose cultures of bone marrow from all patients studied, and a clear increment in cloning was observed with increasing concentrations of Ep. Figure 1 shows the Ep dose-response relationship as a log-log plot for the seven preleukemia patients compared to mean data for five normal subjects. Although colony formation varied widely in the plates without Ep, the mean erythroid colony number in preleukemia cultures was

![Fig. 1. Log-log plot of erythroid colony formation (CFU-E) versus Ep concentration. Data shown for seven preleukemia patients and mean data ± SE for five normals.](image)
greater than twice that of normals in plates containing no added Ep. The difference, however, was not statistically significant within 95% confidence limits ($p = 0.1$) (Table 2). Cloning efficiency differed from patient to patient over the Ep concentrations tested (0.05–2 U/ml), but the slopes of the patients’ Ep dose-response lines were similar to the mean normal slopes.

Figure 2 illustrates the composite Ep dose-response curves for seven preleukemic patients, two patients with erythroleukemia, five patients with untreated polycythemia rubra vera, and five normal volunteers. The mean cloning efficiency in the preleukemia group was greater than control at each Ep dose tested and was significantly different from normal when all data points were considered ($p < 0.02$). If the two patients who did not develop acute leukemia are omitted from the analysis there is no significant difference in cloning efficiency between the normals and preleukemics. Mean colony formation was significantly greater in the polycythemia rubra vera patients compared to the controls at each Ep concentration tested.

Because the Ep dose responses were linear on log-log plot, individual and mean regression lines were constructed. The regression lines had excellent fit, as evidenced by the high correlation coefficients (Table 2). Slopes derived from the Ep dose-response regression lines did not differ significantly between normals and patients with preleukemia and polycythemia rubra vera (Table 2). The mean slope for the erythroleukemia data was substantially less than for the other groups, but because there were only two patients no statement of statistical significance can be made.

Dexamethasone has been shown to potentiate Ep-stimulated erythroid colony formation of normal and polycythemia vera bone marrow in vitro.11,13

![Fig. 2. Log-log plot of composite data on CFU-E in vitro as a function of Ep concentration in seven preleukemia, two erythroleukemia and five polycythemia vera patients and five normal controls. Each data point, mean ± SE](image-url)
Table 3. Effect of Dexamethasone on Erythroid Colony Formation in Preleukemia and Erythroleukemia

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Erythropoietin (1 U)</th>
<th>Erythropoietin (1 U) plus 10^{-9} M Dexamethasone</th>
<th>Augmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>176</td>
<td>212</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>50</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>112</td>
<td>130</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>53</td>
<td>68</td>
<td>28</td>
</tr>
</tbody>
</table>

*Number of erythroid colonies formed per 2 x 10^6 nucleated cells.

At a dexamethasone concentration of 10^{-9} M this augmentation averaged 23% in normals and 21% in polycythemia subjects. Dexamethasone at the same concentration similarly increased the number of erythroid colonies in Ep-stimulated cultures of preleukemia and erythroleukemia marrows (Table 3).

DISCUSSION

Preleukemia is a syndrome of hematopoietic dysfunction preceding the appearance of clinically diagnosable AML. Prominent abnormalities of the erythroid cell line are usually found, and almost all of the patients are anemic. Kinetic studies are consistent with ineffective erythropoiesis similar to that found in AML.

Erythroleukemia (DiGuglielmo syndrome) is characterized by progressive anemia, megaloblastic and dysplastic erythroid hyperplasia with ineffective erythropoiesis, and variable degrees of dysplasia of the granulocytic precursors and megakaryocytes. Erythroleukemia frequently terminates in AML and may be a special case of the preleukemic syndrome. Cytogenetic studies in erythroleukemia and AML suggest that the erythroid and granulocytic precursors are derived from the same neoplastic clone.

Our bone marrow culture studies indicate that erythroid precursor cells in preleukemia and erythroleukemia maintain EP responsiveness in vitro. The mean slope of the Ep dose-response regression lines in preleukemia and polycythemia rubra vera was not statistically different from normal. The linearity of the dose-response relationship and the similarity of the slopes suggest that the “sensitivities” of EP responses may be similar in these patient groups. The observation of increased cloning of marrow cells from patients with preleukemia in cultures not containing added Ep may be related to the patients’ anemia and reflect triggering of erythroid precursors by Ep in vivo. While cloning efficiencies may not be reflective of total erythropoiesis, colony formation noted in some patients with preleukemic and hypercellular bone marrows suggests that the marrow pool of erythroid colony-forming cells is expanded in these cases.

Our data from studies in vitro, showing that in preleukemia and erythroleukemia erythroid precursors are responsive to Ep, are consistent with previous clinical observations. Transfusion of erythroleukemia patients to a normal or high hematocrit caused a marked reduction of Ep excretion and decreased erythroid hyperplasia. The abnormal erythroid morphology, however, was
unaffected. Synthesis of heme by erythroleukemia cells in vitro is also reported to be stimulated by Ep. 18

All of our preleukemia patients were anemic with low reticulocyte counts, and most required frequent red cell transfusions. The majority of these patients had a greater number of erythroid colonies in vitro than did the normal controls. Colony formation in vitro therefore did not correlate with effective erythropoiesis in vivo. The two patients with erythroleukemia also formed erythroid colonies and had a clear erythropoietin dose response. These data obtained in vitro support previous analyses suggesting that ineffective red blood cell production in preleukemia and erythroleukemia leads to anemia, which in turn stimulates Ep production and causes erythroid hyperplasia. 17

Cytogenetic and G-6-PD isoenzyme analyses strongly suggest that preleukemia, AML, chronic myelogenous leukemia, and polycythemia rubra vera are clonal diseases characterized by an abnormal cell line derived from a transformed pluripotent stem cell. 3, 4, 7, 9 It is possible that the Ep modulation noted in vitro with marrow from the preleukemia patients might have reflected stimulation of a normal clone of erythroid precursors while the neoplastic erythroid clone remained Ep unresponsive, but this is unlikely in view of the results of extensive cytogenetic studies performed on two of the patients. In these patients all 135 metaphases examined showed the cytogenetic abnormality of the neoplastic clone. It therefore seems unlikely that an undetected normal clone of erythroid progenitors could account for the Ep dose-response colony formation observed in these patients.

Some neoplastic erythroid proliferation may be autonomous of Ep regulation. For example, Srodes et al. studied two patients with the rare erythroblast crisis of chronic myelogenous leukemia and could not find decreased erythroid activity with hypertransfusion. 22 Also, Friend-virus infection in mice leads to erythropoiesis autonomous of Ep. 23

Although granulocyte-monocyte colony formation is frequently defective in the preleukemia state, 24, 25 our findings suggest that erythroid colony formation in vitro is not similarly impaired.

ACKNOWLEDGMENT

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ERYTHROPOIESIS IN PRELEUKEMIA


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