Decreased Number of Circulating BFU-Es in Paroxysmal Nocturnal Hemoglobinuria

By Bruno Rotoli, Renato Robledo, and Lucio Luzzatto

In order to quantitate early erythroid progenitor cells in paroxysmal nocturnal hemoglobinuria (PNH), we have cultured peripheral blood mononuclear cells from 7 PNH patients in a 0.8% methylcellulose medium containing erythropoietin, 2 U/ml. In our experimental conditions, the number of erythroid colonies obtained per 5 x 10⁶ mononuclear cells plated was 20.1 ± 1.9 (SEM) in normal subjects and 2.8 ± 0.56 (SEM) in PNH patients. In plates from PNH subjects, 38 of 117 showed no growth of erythroid colonies, whereas plates from normal subjects always had colonies. Our findings suggest that PNH patients, despite their hemolytic condition, have a depleted erythroid precursor compartment, and this may play a major role in the pathogenesis of their anemia.

Paroxysmal Nocturnal Hemoglobinuria (PNH) is an acquired blood disorder associated with intravascular hemolysis, which is caused by increased sensitivity of erythrocytes to activated complement fractions. There is evidence that several hematopoietic cell lines are involved. It is still debated whether this disease is to be regarded as primarily myeloproliferative or primarily hypoplastic, since features of both states have been found in PNH.

It is now possible to culture human erythroid cells using semisolid or solid media and appropriate concentration of erythropoietin. By this procedure, early precursor compartment can be quantitated in bone marrow. More recently, circulating progenitor cells (BFU-E) have been detected by cultivating peripheral blood mononuclear cells. We have employed erythroid culture studies from peripheral blood in order to detect a possible quantitative BFU-E abnormality in PNH.

Materials and Methods

Patients

Criteria for the diagnosis of PNH were the following: (A) hemolytic anemia, (B) hemosiderinuria, and (C) a positive Ham test. Three patients were diagnosed in our unit (see significant clinical and hematologic data in Table I), and blood samples from 4 additional patients were obtained from the Blood Transfusion Unit, University Hospital of Milano, by courtesy of Dr. A. Zanella. Preliminary experiments (data not shown) demonstrated that no loss of circulating BFU-Es occurs when heparinised blood is kept at 0–4°C and used within 48 hr.

Cell Preparation and Culture

Peripheral blood (10–30 ml) was placed in Falcon culture tubes containing preservative-free heparin. Mononuclear cells were harvested using a Ficoll-Hypaque technique with slight modifications. Briefly, undiluted blood was layered on an equal volume of Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and centrifuged at 400 g for 40 min at room temperature. Mononuclear cells located at the interface were removed and washed twice in Iscove’s Modified Dulbecco’s Medium (IMDM) (GIBCO, Grand Island, NY). The cell suspension was diluted to about 10⁶/ml in IMDM containing 5% fetal calf serum was incubated at 37°C for 1 hr. Nonadherent cells were removed and washed twice in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 0.8% methylcellulose (Methocel 4000 cps, Dow Chemical Co., Midland, Mich.), 30% fetal calf serum (Euzobio, Paris, France), 1% bovine serum albumin (Sigma, St. Louis, Mo.) prepared according to Tepperman, beta-mercaptoethanol 10⁻⁴ M, and erythropoietin (step III, Connaught Lb., Stillwater, Penna.) 2 U/ml, and distributed in 35 x 10 mm Falcon culture dishes. Cultures were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air.

Scoring of plates was carried out on day 13. The identification of erythroid colonies was performed in situ on the basis of their orange-red color and their characteristic morphology. Cytologic preparations showed typical erythroblasts. A single erythroid colony was composed of subcolonies (from 1 to over 20). Only easily recognizable colonies were scored, counting as one colony the whole ensemble of subcolonies.

Results and Discussion

A marked decrease in the average number of erythroid colonies was observed in PNH patients compared with normal subjects. For each 5 x 10⁶ mononuclear cells plated, we found 2.8 ± 0.56 (SEM) erythroid colonies in PNH patients and 20.1 ± 1.90 (SEM) in normal subjects (Fig. 1). The difference was highly significant by statistical analysis (p < 0.001). Microscopic examination of culture plates showed no evidence of early growth, followed by degeneration. Rather, development of erythroid colonies from PNH peripheral blood mononuclear cells was poor throughout the 13 days of culture. Among four extensively studied subjects (Fig. 2), the two PNH patients...
Table 1. Clinical and Hematologic Data in Three Patients With PNH Analyzed in This Study

<table>
<thead>
<tr>
<th>Patients</th>
<th>F.M.</th>
<th>P.C.</th>
<th>G.G.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/Sex</td>
<td>35/M</td>
<td>25/F</td>
<td>33/M</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>6.1</td>
<td>8.0</td>
<td>8.2</td>
</tr>
<tr>
<td>Blood transfusion requirement (U/mo)</td>
<td>4</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>9</td>
<td>2.6</td>
<td>14</td>
</tr>
<tr>
<td>WBC (x 10^9/liter)</td>
<td>1.5</td>
<td>5</td>
<td>3.1</td>
</tr>
<tr>
<td>Platelets (x 10^9/liter)</td>
<td>150</td>
<td>100</td>
<td>79</td>
</tr>
<tr>
<td>HAM test (% hemolysis)</td>
<td>11</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Hemosiderinuria</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Nocturnal hemoglobinuria</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Infections</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BFU-E/5 x 10^5 cells plated</td>
<td>1.29</td>
<td>2.25</td>
<td>3.12</td>
</tr>
<tr>
<td>SD</td>
<td>±2.56</td>
<td>±2.46</td>
<td>±1.8</td>
</tr>
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</table>

showed a tenfold decrease in BFU-E per plate, and several plates were found without any growth of erythroid colonies. When these results are correlated with the clinical and hematologic parameters (Table 1), it is seen that the patient with the lowest number of BFU-Es/plate was the one with the highest transfusion requirement, the lowest WBC, and a number of serious infectious complications.

A decreased number of CFU-C from bone marrow of PNH subjects was reported in recent studies. However, we have not found any previous data in the literature on erythroid colonies from bone marrow or peripheral blood in PNH patients.

By contrast, in hemolytic anemias other than PNH, it has been reported and we have confirmed (unpublished observations) that there is an increased number of BFU-Es in peripheral blood. Thus, among its many peculiarities, PNH also shows a pattern of hematopoietic precursors unusual for a hemolytic disorder. A number of clinical and hematologic data suggest that hypoproduction of hematopoietic tissue plays an important role in the pathophysiology of this disease: (1) Hyperhemolysis is rarely compensated in PNH patients. In a comprehensive review, it was found that 76% of PNH subjects had Hb below 9 g/dl, with a number of reticulocytes and bone marrow hyperplasia nearly always less pronounced than expected. (2) Decreased number of WBC and platelets is a common finding in PNH: 50% of patients are neutropenic and 70% have less than 1.5 x 10^11/liter platelets. (3) PNH and bone marrow aplasia or hypoplasia are often closely related disorders.

Our results further support the idea that in PNH, qualitative abnormalities are associated with quantitative defects and that a reduction in stem cell compartment, indirectly documented by the decreased number of BFU-Es, is part of the pathophysiology of this disease.
of circulating BFU-Es, is at least in part responsible for the anemia.

NOTE ADDED IN PROOF

Subsequent to acceptance of this paper for publication, an article has appeared by A. Uraba and S. Fujioka (Brit J Haematology 50: 295, 1982) on CFU-E and BFU-E numbers in bone marrow from 8 patients with PNH. These authors found that in six out of eight patients BFU-Es were decreased, in agreement with our findings. On the other hand, in two patients BFU-Es were actually increased, and CFU-Es were very variable. It is possible that because of wide variation in marrow cellularity, sampling of this tissue is less representative than peripheral blood of the total BFU-E compartment.

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