Hematopoietic Progenitors in Cyclic Neutropenia: Effect of Granulocyte Colony-Stimulating Factor In Vivo

By Anna Rita Migliaccio, Giovanni Migliaccio, David C. Dale, and William P. Hammond

The number and growth factor requirements of committed progenitor cells (colony-forming units-granulocyte/macrophage and burst-forming units-erythroid) in three patients with cyclic neutropenia (two congenital, one acquired) were studied before and during therapy with recombinant human granulocyte colony-stimulating factor (G-CSF; 3 to 10 µg/kg/d). When the patients with congenital disease were treated with G-CSF, the cycling of blood cells persisted, but the cycle length was shortened from 21 days to 14 days, and the amplitude of variations in blood counts increased. There was a parallel shortening of the cycle and increase of the amplitude of variations (from two- to three-fold to 10- to 100-fold) in the number of both types of circulating progenitor cells in these two patients. In the patient with acquired cyclic neutropenia, cycling of both blood cells and progenitors could not be seen. In cultures deprived of fetal bovine serum, erythroid and myeloid bone marrow progenitor cells from untreated patients and from congenital disorder and in an acquired form, with essentially identical phenotypic presentations.

Studies of the pathophysiology of cyclic hematopoiesis demonstrate that the abnormality lies in the regulation of cell production, not in peripheral destruction. The demonstration that bone marrow transplantation from a child to her sister with leukemia transferred cyclic neutropenia suggests that it is a stem cell disease. Prior studies of hematopoietic progenitor cells in such patients show that progenitor cell numbers fluctuate cyclically. One recent study suggests that an abnormality in responsiveness to granulocyte-macrophage colony-stimulating factor (GM-CSF) may be a characteristic feature of the progenitor cells in this disease. The availability of purified, molecularly cloned hematopoietic growth factors to promote the proliferation and differentiation of neutrophils in vivo has led to a series of clinical trials in patients with various causes of neutropenia. We have recently reported the efficacy of recombinant human G-CSF.

Fig 1. Normal blood progenitor cells. Numbers of BFU-E (O) and CFU-GM (□) derived colonies per 3 × 10⁶ light density blood cells (A) and per milliliter of blood (B) from one normal volunteer. The absolute number of neutrophils per microliter of blood (B, △) is also reported. Control blood BFU-E and CFU-GM from nine different volunteers were 72 ± 11 and 23 ± 16, respectively (A, △ and △, respectively).

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granulocyte colony-stimulating factor (G-CSF) in ameliorating the neutropenia and diminishing the frequency and severity of infections in patients with cyclic hematopoiesis. In this report, we present data on the progenitor cell growth characteristics of our first three patients in serum-supplemented and in serum-deprived conditions and the effects of in vivo treatment with G-CSF on these progenitors.

MATERIALS AND METHODS

Patients. The clinical features of the three patients, including their response to G-CSF treatment, has been reported previously; the case numbers are the same as in that report. Patients 1 (male, age 14) and 3 (female, age 23) had congenital cyclic neutropenia. Patient 2 acquired cyclic neutropenia at the age of 63. She did not have elevated large granular lymphocytes as in some other acquired cases. Patient 1 received G-CSF at 3 μg/kg/d intravenously (IV) from day 0 to day 36, 10 μg/kg/d IV from day 37 to day 60, and 5 μg/kg/d subcutaneously (SC) from day 61 to day 106. Both patients 2 and 3 were receiving 3 μg/kg/d IV or SC during the treatment period reported in this study.

Blood and marrow sampling. Blood sampling and marrow aspirations were done with informed consent under a protocol approved by the University of Washington's Human Subjects
G-CSF IN CYCLIC NEUTROPENIA

The light density cell fraction was separated by Ficoll-Hypaque (Organon Teknika Co, Durham, NC) density gradient centrifugation (400g for 20 minutes). Adherent cells were removed by two 1-hour incubations of the cell suspension in plastic flasks (75 cm²) containing 2 x 10⁶ cells in 20 mL of IMDM supplemented with 10% (vol/vol) fetal bovine serum (FBS; HyClone, Logan, UT) at 37°C and in 5% CO₂ in air. The blood light density cell fraction (mononuclear cells) was separated as described above for the marrow cells.

Hematopoietic growth factors. The purified recombinant human hematopoietic growth factors used included erythropoietin (Epo),²⁰ G-CSF²² (Amgen, Thousand Oaks, CA), and granulocyte/macrophage (GM)-CSF.²³ Conditioned medium from COS-1 cells transfected with a plasmid designed for expression of human interleukin-3 (IL-3)²³ (Genetics Institute, Cambridge, MA) was used as a source of human IL-3. The specific activity of IL-3 was 10⁶ units/µg of protein (S. Clark, personal communication). The growth factors were used at concentrations of 1.5 units/mL for Epo, 4.5 x 10⁻¹⁰ mol/L for GM-CSF, 2 x 10⁻¹⁰ mol/L for IL-3, and 2 x 10⁻¹² mol/L for G-CSF. These concentrations induce optimal colony growth in FBS-supplemented or in FBS-deprived cultures of marrow cells from normal volunteers.²⁴ In culture of purified progenitor cells from cyclic neutropenic patients before and during the therapy, these concentrations of growth factors induced 100%, more than 80%, and 50% of maximal colony growth in the case of IL-3, GM-CSF, and G-CSF, respectively.²³ Conditioned medium from phytohemagglutinin-stimulated human leukocytes (PHA-LCM) (3%, vol/vol), prepared serum-free, was used as a positive control in FBS-supplemented cultures.²⁴

Colonies. For the FBS-supplemented culture, each 1 mL dish contained the following components in IMDM:²⁶ methylcellulose (0.8% wt/vol, final concentration), β-mercaptoethanol (7.5 x 10⁻³ mol/L), antibiotics (100 U of penicillin, 250 ng of amphotericin B, and 100 µg of streptomycin), a selected heat-inactivated lot of FBS (40%, vol/vol), and 3 x 10⁶ nonadherent marrow cells or 3 x 10⁵ light density cells from the peripheral blood. In the FBS-deprived culture, the FBS was substituted by deionized bovine serum albumin (BSA; 2 x 10⁻³ mol/L), BSA-adsorbed cholesterol (4 µg/mL) and soybean lecithin (12 µg/mL), iron-saturated transferrin (5 x 10⁻⁹ mol/L), insulin (1.7 x 10⁻⁸ mol/L), amphotericin B, and inorganic salts, sodium pyruvate (10⁻⁴ mol/L), and L-glutamine (2 x 10⁻⁴ mol/L), as described.²⁷ All chemicals were from Sigma, St Louis, MO.

Scoring criteria and data analysis. All cultures were established in duplicate. Plates were incubated at 37°C in a humidified incubator with 5% CO₂ in air and scored at 12 to 14 days for the presence of erythroid bursts (from burst-forming unit-erythroid [BFU-E]) and GM colonies (from granulocyte/macrophage colony-forming unit [CFU-GM]). Erythroid bursts (containing ≥ 200 cells) were identified on the basis of their orange-red color. Colonies composed of more than 50 non-erythroid cells were scored as GM colonies. As previously described,²⁸ more than 85% of the colonies detected in FBS-deprived cultures stimulated with Epo or G-CSF were pure erythroid or granulocytic colonies, respectively.

Marrow colony numbers are reported per 3 x 10⁵ nonadherent marrow cells plated. The number of blood progenitor cells was measured in both FBS-supplemented cultures stimulated with Epo and/or PHA-LCM and in FBS-deprived cultures stimulated with GM-CSF, IL-3, and Epo or G-CSF. From the yield of mononuclear cells and the frequency of erythroid bursts and GM colonies per 3 x 10⁵ cells, the number of BFU-E and CFU-GM per milliliter of blood was calculated, assuming that the cloning efficiency did not change during the cycle. Statistical analysis was performed by means of the paired Student's t test.

CELL PREPARATION. Marrow samples were aspirated from the posterior iliac crest into heparinized syringes and diluted 1:1 with Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY), and the marrow cells were suspended with a pipette.

Fig 2C.
RESULTS

Hematopoietic progenitors in peripheral blood. The numbers of erythroid bursts and GM colonies in peripheral blood were measured both in FBS-supplemented and FBS-deprived cultures. Since the difference observed between the values obtained in the two systems was less than 20%, the results have been pooled, and each data point in Figs 1 and 2 represents the mean of the colonies observed in four separate plates. Under FBS-supplemented conditions, rare mixed erythroid and granulocytic colonies (2 to 15 colonies per dish) were occasionally observed, both in culture of normal and cyclic neutropenic blood cells.

In nine normal adults studied concurrently, the number of BFU-E and CFU-GM were 72 ± 11 and 23 ± 16/3 x 10^3 cells, respectively. The number of BFU-E and CFU-GM detected over a period of 20 days in an adult volunteer ranged from 60 to 95 and from 22 to 35, respectively (Fig 1A).

In patient 3, a congenital cyclic neutropenic patient studied before treatment, the numbers of peripheral blood BFU-E and CFU-GM cycled in a synchronous fashion with an estimated periodicity of 21 days (Fig 2C), similar to the oscillations of marrow colony-forming cells previously reported. In patient 2 before therapy, BFU-E also appeared to cycle with about the same amplitude of variation as for patient 3 (Fig 2B). CFU-GM were relatively low and ranged from 5 to 15 colonies per plate without cyclic variation.

During G-CSF treatment, the average number of blood colony-forming cells did not increase significantly, but, at the peak of the cycle, the relative numbers of BFU-E and CFU-GM appeared to cycle with about the same amplitude of variation as for patient 3 (Fig 2B). CFU-GM were relatively low and ranged from 5 to 15 colonies per plate without cyclic variation.

To further understand the kinetics of the increase in circulating progenitor cells with G-CSF therapy, we compared the number of circulating progenitor cells before and 6 hours after G-CSF administration (Table 1). An increase in the absolute number of progenitor cells, as well as neutrophil counts, was observed 6 hours after G-CSF administration for all three patients. However, this increment was statistically significant only for patients 1 and 2.

Cellular composition of marrow specimens. Table 2 compares the yields and cellular composition of marrow specimens from normals and patients before and during treatment with G-CSF. The number of cells aspirated from patients before treatment was within the range of normals; during G-CSF treatment, the yield of cells increased between 2- and 10-fold in the patients. Monocyte concentrations were evaluated by Giemsa stains and confirmed by nonspecific esterase staining. The mean nucleated cell recovery after adherence from the three cyclic neutropenic patients was heterogeneous, but no consistent pattern of variation was detectable. Monocytes were low but not totally eliminated by the adherence procedure; lymphocytes were variably increased as previously reported in cyclic neutropenia.

Hematopoietic progenitors in bone marrow. The numbers of BFU-E and CFU-GM in cultures of nonadherent marrow cells from normal adult volunteers were quite variable. The coefficient of variation in four separate experiments for each of three different marrow donors was on the order of 30% to 40%, and the variation between donors was even larger (Fig 3A and 3B). The cyclic neutropenic patients had BFU-E and CFU-GM within these ranges (Fig 3). The apparent decrease in proportion of marrow progenitor cells in the patients during G-CSF treatment is on the order of magnitude observed in separate experiments in an individual

Table 1. Progenitor Cells and Neutrophil Counts in the Blood of Cyclic Neutropenic Patients Before and 6 Hours After G-CSF Administration

<table>
<thead>
<tr>
<th>Table 1. Progenitor Cells and Neutrophil Counts in the Blood of Cyclic Neutropenic Patients Before and 6 Hours After G-CSF Administration</th>
<th>Erythroid Bursts (±SEM)/mL Blood</th>
<th>GM Colonies (±SEM)/mL Blood</th>
<th>Absolute Neutrophil Counts (cells ± SEM)/L Blood</th>
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</thead>
<tbody>
<tr>
<td><strong>Patient 1 (mean of 4 exp)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Before G-CSF</td>
<td>407 ± 186</td>
<td>551 ± 260</td>
<td>2,050 ± 548</td>
</tr>
<tr>
<td>6 Hours after G-CSF</td>
<td>815 ± 374</td>
<td>1,087 ± 620</td>
<td>3,750 ± 800</td>
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<tr>
<td>% of baseline</td>
<td>187 ± 15*</td>
<td>176 ± 16*</td>
<td>204 ± 24*</td>
</tr>
<tr>
<td><strong>Patient 2 (mean of 3 exp)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Before G-CSF</td>
<td>248 ± 40</td>
<td>47 ± 11</td>
<td>5,000 ± 610</td>
</tr>
<tr>
<td>6 Hours after G-CSF</td>
<td>357 ± 73</td>
<td>61 ± 13</td>
<td>31,040 ± 1,850</td>
</tr>
<tr>
<td>% of baseline</td>
<td>133 ± 7</td>
<td>141 ± 34</td>
<td>836 ± 38</td>
</tr>
<tr>
<td><strong>Patient 3 (mean of 5 exp)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before G-CSF</td>
<td>344 ± 95</td>
<td>205 ± 79</td>
<td>2,980 ± 170</td>
</tr>
<tr>
<td>6 Hours after G-CSF</td>
<td>386 ± 105</td>
<td>266 ± 116</td>
<td>8,140 ± 4,100</td>
</tr>
<tr>
<td>% of baseline</td>
<td>120 ± 12*</td>
<td>139 ± 27</td>
<td>328 ± 83†</td>
</tr>
</tbody>
</table>

*P < .01, compared with pretreatment value on same day.
†P < .05, compared with pretreatment value on same day.
normal donor. However, marrow hematopoietic progenitors were diluted by the increased numbers of mature cells present during treatment with G-CSF (Table 2). It is not possible to calculate from our data the absolute number of marrow progenitor cells, but, since the total marrow yields increased by an average of four- to fivefold during G-CSF treatment, this number is probably increased as well.

**Growth factor responsiveness of progenitor cells.** When nonadherent marrow cells from normal donors are cultured under FBS-deprived conditions, more than one growth factor is required for maximal BFU-E and CFU-GM growth (Figs 4 and 5, top panels). Epo alone sustained formation of only 11% of the maximum number of erythroid bursts under FBS-deprived conditions. The addition of GM-CSF or IL-3 induced the formation of 26% or 69% of the erythroid bursts, respectively, and both growth factors are required to observe maximal burst growth (Fig 4, top panel). Similarly, G-CSF, GM-CSF, and IL-3 by themselves stimulate less than 36% of GM colony growth (Fig 5, top panel). When the factors are added in paired combinations, the increase in GM colonies is at most additive, and only when all three are present is maximal colony formation observed (Fig 5, top panel).

In all three patients before therapy, Epo alone induced 46% of the maximal erythroid burst growth, and addition of GM-CSF or IL-3 was sufficient to induce 63% or 100% of maximal colony growth, respectively (Fig 4, middle panel). These values are significantly different with respect to the corresponding values observed in culture of normal progenitors. The response of CFU-GM to either G-CSF, GM-CSF, or IL-3 in the patients was not statistically different from that observed in the normals (Fig 5). However, the combination of G-CSF and IL-3 or G-CSF and GM-CSF was sufficient to observe 66% or 100%, respectively, of maximal GM colony formation in all three patients.

During the treatment with G-CSF, some changes in growth factor sensitivity of marrow progenitors were observed in the patients (Figs 4 and 5, bottom panels). In particular, the percent of the maximal number of colonies induced by Epo alone, by Epo and IL-3, and by G-CSF and IL-3 during the therapy was not statistically different from that induced by the same combination of growth factors in the normals; in the case of G-CSF + IL-3, the number of GM colonies induced during the treatment was significantly lower than the number of colonies induced by the same combination of growth factors before the treatment ($P < .01$). Furthermore, although the number of GM colonies induced by G-CSF + GM-CSF during the therapy was still significantly higher than the number of colonies induced in the normals, in this case the number of colonies observed was significantly lower with respect to the maximum ($P < .02$).

In one experiment on marrow cultures from patient 1, a neutralizing anti–IL-3 serum (a gift from Dr S. Clark) was added to the cultures stimulated with the different combinations of growth factors. The antiserum reduced at least by 50% the growth of BFU-E induced by Epo and IL-3 and the growth of CFU-GM induced by IL-3, alone or in combination with G-CSF or GM-CSF. The antiserum did not inhibit the maximal growth of BFU-E stimulated either by Epo and GM-CSF or by Epo, GM-CSF, and IL-3. The antiserum also did not inhibit the growth of GM colonies induced by the combination of G-CSF and GM-CSF or by the combination of G-CSF, GM-CSF, and IL-3.

**DISCUSSION**

Granulocyte colony-stimulating factor controls in vitro the activation and late maturation stages of neutrophil development and synergizes with GM-CSF or IL-3 in inducing proliferation of primitive hematopoietic progenitor cells. We have recently reported that G-CSF administration ameliorates the neutropenia of patients with cyclic neutropenia and serves as an effective therapy for this
Fig 3. Bone marrow progenitor cell numbers. Relative numbers of BFU-E (A) and CFU-GM (B) in cultures of nonadherent marrow cells of patients 1, 2, and 3 before and during treatment with G-CSF. The cultures were performed under FBS-supplemented conditions stimulated with Epo (1.5 units/mL) and/or PHA-LCM (3%, vol/vol). The marrow was aspirated at days -5, 15, 32, and 56 from patient 1, at days -57 and 24 from patient 2, and at days -8, 20, 48, and 55 from patient 3. The data obtained from patients 1 and 3 in three sequential experiments performed during the therapy have been pooled and presented as mean (±SD). The mean numbers (±SD) of BFU-E and CFU-GM obtained in four separate experiments from each of three normal donors (Normals 1, 2, and 3) obtained concurrently are shown for comparison. The relative frequencies (±SD) of BFU-E and CFU-GM obtained from 19 different normal adult volunteers were 53 ± 23 and 67 ± 32, respectively, as indicated by the shaded area.

disease. The present studies were designed to determine the effect of G-CSF on the numbers and characteristics of hematopoietic progenitors in the peripheral blood and marrow, and to determine if this therapy affects both granulocyte and erythroid progenitor cells.

During chronic treatment with G-CSF, the number of progenitor cells in circulation increased, and each injection of G-CSF induced an acute rise in progenitor cells in the blood, findings consistent with those previously reported. The increase was larger for CFU-GM than for BFU-E. In the patients with the congenital disease treated with G-CSF, the cyclic fluctuations in the numbers of circulating BFU-E and CFU-GM persisted with a 14-day periodicity paralleling the change in periodicity of the reticulocyte and neutrophil counts in the blood (Fig 2). Since cycling of blood cell counts continued in these patients, the persistence of regular fluctuations of 20- to 100-fold in blood progenitor cell numbers suggests that the blood cell compartment provides a quantitative reflection of the underlying bone marrow disorder.

Fig 4. Growth factor response of marrow BFU-E. Relative frequencies of BFU-E in nonadherent marrow cells from normals or patients after treatment. For each of the different panel. The data represent the mean (±SEM) of three separate experiments for the normals and the patients before the therapy and five separate experiments for the patients during the therapy. The data are expressed as percent of colony numbers observed in the dishes stimulated with Epo, GM-CSF, and IL-3 (100% = 24 ± 3, 77 ± 11, and 32 ± 4 BFU-E per 3 x 10⁴ nonadherent marrow cells in cultures of normals or patients before and during therapy, respectively). Statistically significant differences (analyzed by student’s t test) between patients and normals are indicated by asterisks (*, 2P < .05; **, 2P < .01).
Although each injection of G-CSF induced an acute rise in progenitor cells in the blood, this fact alone cannot explain the regular changes observed in both periodicity and amplitude of fluctuations in the circulating progenitor cells in the patients with the congenital disease. We believe that these variations reflect an action of G-CSF on the hematopoietic stem cell, as suggested by Ikebuchi et al on the basis of in vitro experiments. 

In FBS-supplemented conditions, the addition of individual hematopoietic growth factors is sufficient to induce maximal colony formation. In contrast, in FBS-deprived conditions, each individual growth factor is a poor stimulus to colony formation, and maximal growth of erythroid bursts and GM colonies is induced only by the combination of Epo, GM-CSF and IL-3 or G-CSF, GM-CSF and IL-3, respectively. The proportion of all colony forming cells that can be stimulated to form colonies under FBS-deprived conditions by a particular combination of growth factors is very stable in normals. These results are generally interpreted in terms of a hierarchy of progenitor cells that progressively respond to IL-3, GM-CSF, and G-CSF and that distinguish two criteria of growth factor responsiveness of progenitor cells: The first is represented by the concentration of growth factor that induces 50% of plateau colony growth, and the second is represented by the proportion of the plateau number of colonies induced by a growth factor with respect to the maximal number of colonies detectable under FBS-deprived culture. While the first parameter reflects the efficiency of the growth factor receptors to trigger cellular proliferation, the second is an indication of how deeply a growth factor may recruit cell differentiation in the hierarchy of progenitor cells. It has been recently published that CFU-GM from cyclic neutropenic patients differ from normal progenitors in terms of the concentration of GM-CSF, which induces 50% of maximal colony growth in FBS-supplemented cultures. Follow-up studies using purified progenitor cells have confirmed these data and established that progenitor cells from cyclic neutropenia patients are 5 to 10 times less responsive to G-CSF than are normal progenitors, while their response to IL-3 is normal. 

We investigated whether the maximal number of colonies induced in these patients by combinations of growth factors, in particular GM-CSF, in FBS-deprived cultures would also be different from the normals. Differences were observed between progenitor cells from normals and cyclic neutropenic patients in terms of growth factor sensitivity in serum-deprived cultures. In particular, maximal marrow CFU-GM colony formation in the patients occurred with G-CSF plus GM-CSF, with or without the addition of exogenous IL-3 (Fig 5). This fact is even more noteworthy since the concentrations of GM-CSF and G-CSF were suboptimal for cyclic neutropenic progenitor cells. One possible explanation of this finding is that bone marrow cells before therapy, harvested when the patients were neutropenic, were intensely stimulated in vivo by endogenous factors. However, the treatment with G-CSF abrogated the neutropenia but did not change the GM-CSF + G-CSF responsiveness. Alternatively, accessory cells, which represent more than 99% of the cells plated, might release IL-3 in the culture. As a first attempt to prove this hypothesis, an anti-IL-3 serum was added to the serum-deprived cultures. The antisera failed to block the maximal colony formation induced by only two factors in patient 1, suggesting that in vitro production of IL-3 is an unlikely explanation. However, additional experiments using anti-sense oligonucleotides for
onset cyclic neutropenia is associated with increased large granular lymphocytes. J Exp Med 164:2089, 1986

An important question raised by treatments of patients with hematopoietic growth factors has been whether these agents cause depletion or progressive loss of cells from the progenitor cell compartments and whether they "prime" progenitor cells to respond more readily to this or other growth factors, as suggested by in vitro studies and as well as by in vivo trials on primates. The long-term data from these patients, in particular, from patient 1 now treated for more than 2 years with daily G-CSF, show that this does not occur. In fact the numbers of blood and marrow progenitors did not decrease, and if a change in growth factor sensitivity occurred in these patients during the therapy, this change was toward the normal response. Longer follow-up with serial marrow and blood studies will be required to determine if there are long-term effects of this potent therapy.

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