Colony-stimulating Factor in Patients With Chronic Neutropenia

By John R. Wewerka and David C. Dale

Urinary and serum colony-stimulating factor (CSF) levels were measured in 11 patients with chronic idiopathic neutropenia without infections and in 10 normal individuals. Urinary CSF output was determined using mouse marrow target cells, and serum CSF activity was assayed with human marrow target cells by the double agar layer technique. Using these methods, there was no significant difference between CSF levels of neutropenic and normal subjects. These data indicate that CSF levels are not inversely related to the blood neutrophil count in chronic idiopathic neutropenia and suggest that CSF is not a hormone regulating the blood neutrophil count in a manner analogous to the erythropoietin regulation of circulating erythrocyte levels.

Bone marrow granulocytic precursor cells will form colonies of granulocytes and macrophages in a tissue culture system in vitro if a specific stimulating substance is present. This substance, colony-stimulating factor (CSF), has been derived from conditioned medium of cell cultures, cell feeder layers, human serum, and human urine. The culture system provides an assay for quantifying levels of CSF in human urine and serum. The role of CSF in the regulation in vivo of granulocytopoiesis is still unclear. In particular, it has not been shown conclusively whether CSF levels are regularly increased in patients with neutropenia similar to the increase of the erythropoietin levels in most patients with anemia.

Chronic neutropenia is characterized by long-standing stable blood neutrophil counts of less than 2000 neutrophils/cu mm associated with relatively infrequent infections. This disorder provides a clinical model for investigating neutrophil regulation. The present study examines the relationship between the blood neutrophil count, the urinary CSF output, and the serum CSF concentration to determine whether CSF levels are increased in chronic neutropenic patients without infections.

MATERIALS AND METHODS

Subjects

The 11 patients studied had neutrophil counts of less than 2000/cu mm of greater than 1-yr duration (Table I). The etiology of the chronic neutropenia in all patients was unknown. One patient (A.C.) had a healing perirectal inflammation and a positive test for rheumatoid factor but no other evidence for Felty's syndrome; another (D.S.) had a barely palpable spleen, a positive test for hepatitis antigen and mild thrombocytopenia, but normal liver function tests. Patient P.C. had mild iron-deficiency anemia. Otherwise, the patients were entirely well, without anemia, thrombocytopenia, splenomegaly, or any signs of infection or inflammation. Cyclic neutropenia
Table 1. Clinical and Laboratory Data for 11 Patients With Chronic Neutropenia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Duration (yr)</th>
<th>WBC/cu mm (Range)</th>
<th>PMN/cu mm (Range)</th>
<th>Monocytes/cu mm (Range)</th>
<th>Colonies/Dish With Serum</th>
<th>Colonies/Dish With Urine†</th>
<th>CSF Output (units/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.C.</td>
<td>7/M</td>
<td>7</td>
<td>3400-3600</td>
<td>0-34</td>
<td>864-884</td>
<td>28</td>
<td>58, 82, 61</td>
<td>127, 296, 151</td>
</tr>
<tr>
<td>C.P.</td>
<td>22/M</td>
<td>6</td>
<td>2800-3100</td>
<td>0-102</td>
<td>1024-1700</td>
<td>56</td>
<td>13, 17, 13, 33</td>
<td>76, 123, 110, 257</td>
</tr>
<tr>
<td>V.B.</td>
<td>14/F</td>
<td>3</td>
<td>2400-2900</td>
<td>168-297</td>
<td>120-416</td>
<td>49</td>
<td>26, 8, 21, 14</td>
<td>174, 103, 129, 89</td>
</tr>
<tr>
<td>A.M.</td>
<td>58/F</td>
<td>13</td>
<td>3500-4000</td>
<td>60-420</td>
<td>280-525</td>
<td>59</td>
<td>54, 13, 24</td>
<td>533, 511, 605</td>
</tr>
<tr>
<td>C.T.</td>
<td>16/F</td>
<td>5</td>
<td>2700-3500</td>
<td>64-609</td>
<td>348-744</td>
<td>61</td>
<td>102, 112, 24</td>
<td>444, 586, 657</td>
</tr>
<tr>
<td>A.C.</td>
<td>45/M</td>
<td>2</td>
<td>5000-7300</td>
<td>284-810</td>
<td>511-1242</td>
<td>94</td>
<td>73, 92, 83, 91</td>
<td>900, 1751, 1315, 1510, 1249, 708, 564, 564</td>
</tr>
<tr>
<td>L.D.</td>
<td>60/F</td>
<td>18</td>
<td>2500-2900</td>
<td>290-1102</td>
<td>250-660</td>
<td>44</td>
<td>59, 44, 56, 70</td>
<td>486, 259, 399, 1219</td>
</tr>
<tr>
<td>D.S.</td>
<td>23/M</td>
<td>1</td>
<td>2300-3000</td>
<td>437-1147</td>
<td>30-341</td>
<td>47</td>
<td>33, 14, 3, 7</td>
<td>584, 205, 123, 219</td>
</tr>
<tr>
<td>G.H.</td>
<td>58/F</td>
<td>4</td>
<td>2200-3200</td>
<td>704-1333</td>
<td>279-663</td>
<td>53</td>
<td>40, 28, 45, 9</td>
<td>325, 182, 310, 104</td>
</tr>
<tr>
<td>E.W.</td>
<td>49/F</td>
<td>10</td>
<td>3300-4100</td>
<td>770-1722</td>
<td>328-532</td>
<td>38</td>
<td>87, 94, 62, 75</td>
<td>730, 671, 472, 482</td>
</tr>
</tbody>
</table>

*Values shown are mean numbers of colonies per dish for duplicate cultures using one serum sample.
†Values shown are mean numbers of colonies per dish or units per day for individual 24-hr urine samples. These values are the means for quadruplicate cultures.

had been excluded in all cases by sequential neutrophil counts. The normal subjects were ten healthy volunteers with normal physical examinations and normal hematologic values (Table 2).

**Blood counts.** Total and differential leukocyte counts were made for each individual on samples collected at 8 a.m. for each study day using standard methods.

**Urinary CSF.** Twenty-four-hour urine samples were collected for 3–6 consecutive days from each patient. In 4 normal individuals, 24-hr collections were made for 3 consecutive days and in 6 others for one 24-hr period. The urine was collected in polypropylene screw-top bottles and immediately frozen at −20°C. After measuring the 24-hr urine volume, an aliquot of 200–400 ml was taken from each sample and centrifuged at 2000 g for 10 min at 4°C to remove cellular debris. The supernatant urine was then concentrated tenfold in an Amicon ultrafiltration chamber using a Diaflo UM10 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). The urine concentrates and, in some cases, the filtrates were dialyzed in Visking tape (Union Carbide Corp., Chicago, Ill.) against 2 liters of distilled water per sample over 72 hr at 4°C with two changes of dialysis fluid. Considerable sample volume increases occurred during dialysis and therefore each sample volume was measured and recorded after dialysis. The specimens were then spun at 2000 g for 10 min at 4°C to remove any precipitate and the supernatant solutions filtered through 0.45-μm Millipore membranes. All samples were stored in polypropylene tubes at −20°C until assayed for CSF.

Urinary CSF activity was measured by a modification of the mouse bone marrow culture system in which methylcellulose was substituted for agar as the supporting medium. For each assay, 0.75 ml of the material to be tested was added to a polypropylene tube containing 2.5 ml of 2% methylcellulose, 1.0 ml modified McCoy's medium, 0.5 ml horse serum, 0.25 ml bovine serum albumin, and 0.5 ml modified McCoy's medium containing 7.5 x 10⁵ C57Bl mouse bone marrow cells. The resulting suspension was thoroughly mixed, and then 1.1-ml aliquots were pipetted into four tissue culture dishes to give a marrow cell concentration of 75,000 nucleated cells per dish. The cultures were incubated at 37°C in a humidified incubator with a continuous flow of 8% CO₂ in air. After 7 days, colonies of 50 or more cells were counted using an inverted microscope. CSF activity was determined for the dialyzed and filtered urine samples by assaying...
Table 2. Laboratory Data for 10 Normal Subjects

<table>
<thead>
<tr>
<th>Normal Volunteers</th>
<th>WBC/cu mm (Range)</th>
<th>PMN/cu mm (Range)</th>
<th>Monocytes/cu mm (Range)</th>
<th>Colonies/Dish With Serum*</th>
<th>Colonies/Dish With Urine†</th>
<th>CSF Output (units/day)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.S.</td>
<td>3900-4400</td>
<td>2021-2691</td>
<td>195-396</td>
<td>50</td>
<td>88, 100, 72</td>
<td>680, 643, 428</td>
</tr>
<tr>
<td>M.S.</td>
<td>4100</td>
<td>2091</td>
<td>236</td>
<td>N.D.</td>
<td>20</td>
<td>677</td>
</tr>
<tr>
<td>D.R.</td>
<td>5100-6000</td>
<td>2240-3360</td>
<td>357-480</td>
<td>100</td>
<td>12, 15, 9</td>
<td>95, 235, 90</td>
</tr>
<tr>
<td>P.W.</td>
<td>5200</td>
<td>2444</td>
<td>208</td>
<td>N.D.</td>
<td>35</td>
<td>557</td>
</tr>
<tr>
<td>C.G.</td>
<td>5700</td>
<td>2907</td>
<td>275</td>
<td>37</td>
<td>12</td>
<td>332</td>
</tr>
<tr>
<td>D.L.</td>
<td>6300-7800</td>
<td>2961-3882</td>
<td>252-468</td>
<td>51</td>
<td>59, 55, 81</td>
<td>246, 235, 443</td>
</tr>
<tr>
<td>J.E.</td>
<td>6600</td>
<td>3036</td>
<td>396</td>
<td>79</td>
<td>47</td>
<td>1205</td>
</tr>
<tr>
<td>A.S.</td>
<td>6100</td>
<td>3416</td>
<td>0</td>
<td>60</td>
<td>30</td>
<td>339</td>
</tr>
<tr>
<td>C.W.</td>
<td>6000-7000</td>
<td>3456-5110</td>
<td>192-660</td>
<td>32</td>
<td>41, 29, 35</td>
<td>329, 445, 478</td>
</tr>
<tr>
<td>T.G.</td>
<td>8600</td>
<td>4042</td>
<td>602</td>
<td>27</td>
<td>27</td>
<td>636</td>
</tr>
</tbody>
</table>

N.D., not done.

*Values shown are mean numbers of colonies per dish for duplicate cultures using one serum sample.
†Values shown are mean numbers of colonies per dish or units per day for individual 24-hr urine samples. These values are the means for quadruplicate cultures.

Serial dilutions (undiluted, 0.5 × 0.33 × 0.25 × 0.20 ×, and 0.165 × ). The serial dilutions for four or five urine samples were tested together with similar dilutions of a stable L-cell conditioned medium standard in each experiment. The urine filtrates derived from the ultrafiltration procedure were also assayed similarly. The following equation based on the formula of Stanley was used to determine the daily urinary CSF output for each 24-hr sample:

\[
\text{CSF output/day} = \frac{V_u \times B_u \times \alpha_u}{CF \times A_u \times \alpha_L \times 0.15},
\]

Where:
- \( V_u \) = 24-hr urine volume in milliliters
- \( A_u \) = urine volume after concentration in milliliters
- \( B_u \) = urine volume after dialysis in milliliters
- \( CF \) = concentration factor
- \( \alpha_u \) = effective CSF concentration of the test urine
- \( \alpha_L \) = effective CSF concentration of the standard L-cell conditioned medium
- 0.15 = sample volume tested per dish in milliliters

The concentration factor was 10 in all ultrafiltration procedures. The values of \( \alpha_u \) and \( \alpha_L \) were estimated with a computer using the SAAM program prepared by Berman and Weiss. The computation involved a nonlinear least-squares fitting of the formula \( K[1 - (1 + \alpha \cdot t) \exp \alpha \cdot t] \) derived by Maritz et al., where \( K \) is the expected number of potential colony-forming cells, \( t \) is the assay dilution of the CSF preparation, and \( \alpha \) is the effective concentration of CSF before dilution for assay.

Protein concentrations for the dialyzed and filtered urine samples were determined by the method of Lowry et al. for calculation of the CSF outputs per milligram urinary protein for each urine sample.

**Serum CSF**

Serum CSF activities were determined by the double agar layer technique of Mintz and Sachs. Venous blood samples, collected at 8 a.m. on one of the days of urine collection, were allowed to clot at room temperature for 2-4 hr; the serum was separated by centrifugation, and then frozen at -20°C. All samples were tested in a single assay against the same bone marrow cells. The bone marrow target cells were obtained after mastectomy from a breast cancer patient who had no evidence of metastatic disease and who had not received radiotherapy or chemotherapy. Her peripheral blood counts and bone marrow smears were normal. The bone marrow cells were allowed to sediment in a heparinized glass tube for 1.5 hr and were then centrifuged. The buffy coat was removed and suspended in McCoy's plus medium without fetal calf serum. Tissue culture dishes were prepared that contained an underlayer of 0.375 ml test serum and 1.125 ml McCoy's medium with 20% fetal calf serum. Over this layer were plated 1 x 10⁵ nucleated marrow cells.
(omitting metamyelocytes, stab forms, and mature granulocytes) in 1.5 ml of McCoy’s medium with 0.5% agar and 20% fetal calf serum. A control assay without serum was performed. After 10 days of incubation colonies of 50 or more cells were counted. Approximately 90% of the cells in these colonies were band or segmented neutrophils. The results were expressed as mean numbers of colonies for duplicate culture dishes for each serum sample.

RESULTS

The blood leukocyte, neutrophil, and monocyte counts and the serum and urine CSF levels for the 11 patients and 10 normal subjects are shown in Tables 1 and 2. For the 10 normal volunteers the mean urinary CSF output was 519 ± 93 (1 SEM) per day or 456 ± 109/g protein excreted/day. For the 11 neutropenic patients, the CSF outputs were 427 ± 96/day or 372 ± 39/g protein excreted/day. There was no significant difference between the two groups for the mean values of CSF output per day or CSF output/mg protein/day (p > 0.20, Student’s t test). The CSF activity also was expressed as the number of colonies formed per plate for a 1:1 dilution of the dialyzed, filtered urine (Tables 1 and 2). The urine filtrates were found to contain no or only very minimal CSF activity. The serum CSF activities for the 8 normal subjects were 54 ± 8 colonies/dish and for the 11 neutropenic subjects, 51 ± 5 colonies/dish; there was no significant difference in these values (p > 0.20, Student’s t test). No colonies were formed in the control assay without the addition of human serum. There was no association between the urine CSF output and the serum CSF levels by the Spearman rank correlation test (p = –0.143, p > 0.20).

DISCUSSION

The observation that colony-stimulating factor will cause granulocytic precursor cells to form colonies in vitro has prompted many investigators to study the possible role of CSF as a regulator of granulocytopoiesis in vivo. The injection of a partially purified CSF material derived from human urine into mice has been shown to elevate the granulocyte count. In patients with chronic granulocytic leukemia, serum and urinary CSF levels generally are elevated, normal, or decreased in acute leukemia, depending, at least in part, on the stage of the disease. In other clinical and experimental situations, evidence has accumulated to suggest a relationship of the blood granulocyte count and CSF levels. In cyclic neutropenia in dogs and man, both urine and serum CSF levels vary cyclically, the highest levels of CSF occurring when the blood neutrophil count is maximally reduced. In animals made neutropenic with irradiation, methotrexate, antineutrophil serum, and endotoxin, rapid increases in CSF levels have been observed. Infections, which presumably increase granulocyte utilization, raise CSF levels in both patients and experimental animals. Endotoxin administration in man also raises serum CSF levels. Hydrocortisone, which may reduce granulocyte turnover, is reported to lower rapidly serum CSF levels in mice.

Despite these observations, considerable controversy persists concerning the interpretation of CSF levels in serum and urine. In particular, it remains to be demonstrated conclusively that changes in the levels of blood granulocytes are directly related to changes in CSF levels. In cyclic neutropenia, the rise in CSF levels corresponds temporally to the period of increased granulocytopoiesis but it also occurs at the time when fever and infections most often are observed.
The CSF response to irradiation-induced neutropenia does not occur in germ-free mice, although these animals can increase their serum CSF levels in response to endotoxin administration. When mice are made neutropenic with cyclophosphamide without reducing the granulocyte counts to zero, increased CSF is not observed. Phytohemagglutinin, concanavalin A, and pokeweed mitogen will release CSF from lymphocytes, suggesting that increased CSF levels can occur in immunologic reactions in which granulocytes are not primarily involved. Thus the evidence that CSF is a prime regulator of granulopoiesis is at present tentative and indirect.

The present study adds further evidence to indicate that CSF levels are not necessarily inversely related to the blood granulocyte count. In patients with chronic idiopathic neutropenia without infections, CSF levels in urine and serum were normal. The fact that 9 of 11 patients studied had neutrophil counts of less than 500/cu mm during the study period emphasizes that this relationship exists even when the neutropenia is severe. These findings are similar to those of a previous report in which 3 of 4 patients with neutrophil counts of less than 1000/cu mm had normal CSF levels, presumably in the absence of infections. Since the levels of CSF were normal, it is unlikely that reduced CSF production is a prime defect in these patients. It may also be noteworthy that the elevated blood monocyte counts in these patients was not associated with heightened CSF values, even though blood monocytes may be a principal source for endogenous CSF.

At the present time, however, interpretation of these and previous clinical measurements of CSF must be made with considerable caution. During the period that clinical investigations of CSF have been made, it has been increasingly appreciated that many problems exist in the assay methods. The significance of CSF activity in human urine assayed against mouse cells is somewhat uncertain. Human urinary CSF can be measured only in an assay using mouse marrow, and only some mouse marrow precursor cells are stimulated by human urinary CSF. In cultures using normal and leukemic human leukocyte feeder layers, mouse marrow growth is stimulated by feeder layers which fail to stimulate human marrow growth. Also, a low-molecular-weight CSF can be isolated from human leukocyte conditioned media which will stimulate human marrow but not mouse marrow. In addition, urinary CSF does not appear to be simply a filtration product from serum CSF. Thus it is doubtful that the urine and serum-stimulating factors assayed in this study are, indeed, the same substance and some authorities now even question if urinary CSF has any physiologic significance.

The serum CSF assay employed, using human marrow and the double agar layer technique, permits reproducible serum activity measurements, but there are important problems with this assay. Although the marrow cells in the present study have been plated at a concentration which was not autostimulatory, it is conceivable that the serum activities measured were the indirect effect of the serum on CSF-producing cells in the culture plates. The double layer technique minimizes the inhibitory effects of certain poorly diffusible lipoprotein substances which may be present in serum, but it is also possible that the mature granulocytes or other cells present in the marrow samples could inhibit colony growth. Finally, although the 24-hr urinary CSF output values may
reflect daily CSF production in these patients, the serum levels do not necessarily reflect CSF production and utilization in these subjects. Despite these concerns, the data presented in this report strongly suggest that neutropenia alone is not a stimulus to raise serum or urine CSF levels.

REFERENCES


27. Chan SH, Metcalf D: Local and systemic control of granulocytic and macrophage pro-
CSF IN CHRONIC NEUTROPENIA


Colony-stimulating factor in patients with chronic neutropenia

JR Wewerka and DC Dale