Colony-stimulating Factor and Inhibitor Levels in Acute Granulocytic Leukemia

By Donald Metcalf, Soh Ha Chan, Frederick W. Gunz, Paul Vincent, and Robert B. M. Ravich

Assays were performed on bone marrow colony-stimulating-factor (CSF) levels in the serum and urine of 33 patients with acute granulocytic and myelomonocytic leukemia. Of 251 serum samples, 30% showed elevated CSF levels as did 53% of 1422 urines tested. Levels of serum inhibitors of colony formation were abnormally low or undetectable in 57% of sera from leukemic patients. Patients in relapse were unable to respond to infections by developing high serum CSF levels and low serum-inhibitor levels were more common in patients with a short survival time and in some types of patient during relapses.

Bone marrow cells are able to proliferate in agar cultures and form colonies of granulocytes and/or macrophages if stimulated by the colony-stimulating factor (CSF). Because a sigmoid dose-response relationship exists between colony numbers and CSF concentration, bone marrow cultures can be used to assay CSF levels in biological fluids. CSF is detectable in the serum and urine of normal mice and humans and appears to be a normal regulator of granulopoiesis and monocyte formation. Studies on CSF in human urine have indicated that it is probably a glycoprotein of molecular weight approximately 45,000. Detection of colony-stimulating activity in serum is often masked by the presence of lipoprotein inhibitors, which block the action of CSF in vitro. These inhibitors can be precipitated by dialysis of the serum, and assays have revealed that normal human sera possess uniformly high inhibitor levels.

Preliminary surveys on sera and urine from patients with various types of leukemia indicated that CSF levels were higher than normal in some patients and sequential studies on urine from such patients have shown fluctuations in CSF output during the course of the disease. The present study on patients...
with acute granulocytic leukemia was undertaken to determine CSF levels in
the serum and urine and the level of serum inhibitors of colony formation
during the course of this disease. Because of the extensiveness of the data, this
initial paper presents only a general overall summary of the results. A sub-
sequent paper will discuss individual patients in detail and the influence of
various types of therapy on CSF and inhibitor levels.

**Materials and Methods**

**Collection and Preparation of Material**

Sera and urine specimens were collected from a total of 33 patients age 14–70 yr in the
Royal Melbourne Hospital and the Clinical Haematology Unit of the Kanematsu Memorial
Institute, Sydney Hospital. Twenty-four-hour urine collections were made in plastic
bottles containing 2 ml of 20% sodium azide. The sera and samples of each urine from
Sydney patients were flown to Melbourne for assay at weekly intervals. Portions (2 ml)
of each serum sample were dialyzed in Visking dialysis tubing (wall thickness 0.001 in).
again three changes of 500 ml of distilled water at 4°C for 3 days. Precipitates formed
during dialysis and containing the serum inhibitors were removed by centrifugation before
Millipore filtration of the dialyzed serum. Assays for colony-stimulating activity were
performed on the dialyzed serum and assays for inhibitor levels on undialyzed portions of
the same serum sample. Urine specimens were measured and 40-ml samples dialyzed in
Visking dialysis tubing against three changes of 1000 ml of distilled water at 4°C for 3
days. Urine specimens were then centrifuged and the supernatant urine was Millipore-
filtered and assayed for colony-stimulating activity.

**Bone Marrow Culture Assays**

All assays for CSF were performed using cultures of pooled bone marrow cells from 3-mo-
old C57BL mice. The technique and culture media used in the agar culture of mouse bone
marrow cells have been described in full recently. All cultures were performed in 35-mm
plastic Petri dishes (Falcon Plastics, Los Angeles) using volumes of 1 ml of 0.3% agar in
modified Eagle’s medium, containing 75,000 bone marrow cells. Serum or urine samples to
be assayed were pipetted into replicate culture dishes before the addition of agar medium
containing bone marrow cells. Sera were assayed using volumes of 0.1 and 0.05 ml and
urine samples using 0.15-ml volumes. The assay samples were mixed thoroughly with the
agar medium before gelling occurred and the cultures incubated for 7 days at 37°C in a
fully humidified atmosphere of 10% CO₂ in air. Colony counts were performed at × 25
magnifications using a dissecting microscope with indirect lighting, scoring all aggregates
larger than 50 cells as colonies. Assay runs included cultures stimulated by two standard
human urine pools and two standard serum pools and colony counts were standardized on
the basis of colony counts obtained with these pools. Colony-stimulating activity was ex-
pressed as the number of colonies stimulated by 0.1 ml of serum or 0.15 ml of urine. The
24-hr output of CSF in the urine was expressed as the number of colonies stimulated by
0.15 ml of standardized urine by applying a correction factor: 24-hr-urine volume in
ml/1000 ml.

Because of the sigmoid relationship between colony counts and CSF concentration, the
assay of unconcentrated urine specimens can lead to underestimates if CSF concentrations
are low. Similarly, because of the limiting incidence of in vitro colony-forming cells in the
bone marrow (2/10³ bone marrow cells), colony counts in individual cultures cannot ex-
ceed 150. With very high CSF concentrations seen in occasional leukemic urines the con-
centration of CSF exceeds that needed to stimulate colony formation by all available cells
and an underestimate of CSF results unless serial dilutions of the urine are assayed. In the
present mass survey, neither concentration nor titration procedures were used and
colony counts below 5 or above 120/0.15 ml were probably underestimates of CSF levels.

Assays for inhibitor levels in serum samples were performed in duplicate, mixing 0.1 ml
of the test undialyzed serum in the empty culture dishes with 0.05 ml of a standard pool of
dialyzed human serum of high colony stimulating activity. Agar medium containing bone
marrow cells was then added and, after incubation, colony counts were performed.
Inhibitor levels were expressed as the mean percentage reduction in colony counts in the
test cultures compared with colony counts in the cultures of the active pooled human
serum mixed with 0.1 ml of normal saline.

Table 1.—Summary of CSF and Inhibitor Levels in Leukemic Patients
Included in Survey

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age and Sex</th>
<th>Duration of Disease (months)</th>
<th>Number of Urines Assayed</th>
<th>Number With Elevated CSF</th>
<th>Number of Sera Assayed</th>
<th>Number With Elevated CSF</th>
<th>Number of Sera with Subnormal Inhibitors</th>
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AGL, acute granulocytic leukemia; AUL, acute undifferentiated leukemia; CML → AGL, acute terminal phase of chronic granulocytic leukemia; AMML, acute myelomonocytic leukemia.

*Duration of disease from diagnosis or acute transformation to death. Patients still alive indicated by 8+, and so forth.
Clinical Assessment of Patients

Patients were classified as being either in relapse or in partial or complete remission. Classification was made on clinical and hematological grounds, the criteria used being those of Bisel. Infections in leukemic patients were assessed on the basis of fever (38°C or above), positive bacteriological cultures and response to antibiotics. Three of the 33 patients studied were diagnosed as acute myelomonocytic leukemia, three as being in the acute blastic phase of chronic myeloid leukemia, and three as acute undifferentiated leukemia. The findings in these patients were similar to those in the remainder of the group and have been included in the general data from the patients with acute granulocytic leukemia. All assays were performed without knowledge of the clinical or hematological status of the patient and the two sets of data were collated independently.

Results

General

In total, CSF levels were assayed in 1422 urine samples and CSF and inhibitor levels in 251 serum samples. Table 1 lists the number of observations made on individual patients, and it may be seen that where sufficient samples were analyzed a reasonable uniformity in the pattern of results was observed between individual patients.

Using the present techniques, normal limits for daily urine CSF output have been established from 99 subjects as 0-40 colonies/0.15 ml. Normal serum CSF levels were determined on dialyzed serum from 65 normal adults and from the data (Fig. 1) limits of 3-20 (mean 10) colonies/0.1 ml were established. Inhibitor levels in 98% of 187 undialyzed normal sera were found to lie between 50 and 100%, and a level of 50% was taken as the lower normal limit. These normal limits are indicated by lines in the figures to follow.

Serum CSF and Inhibitor Levels

Assays were performed on 251 samples from 26 patients, the time interval covered in individual patients ranging from one day to 12 mo. The incidence of sera with elevated levels of CSF in individual patients is shown in Table 1. For 245 of these sera, paired analyses were made of CSF and inhibitor levels and these data are shown graphically in Fig. 2.

From the data in Fig. 2 it may be seen that 74/245 (30%) of leukemic sera had elevated CSF levels, the highest values being 10 times the upper normal limit. Conversely 18/245 (7%) had levels below the normal limit, colony stimulating activity being undetectable in 11 sera.

In agreement with an earlier study, inhibitor levels in leukemic sera showed a bimodal distribution, 92 being within normal limits, whereas in 153/245
CSF AND INHIBITORS IN LEUKEMIA

Fig. 2.—Colony-stimulating activity plotted for 245 leukemic sera (mean number of colonies stimulated by 0.1 ml dialyzed serum) vs. inhibitor levels (mean per cent reduction of colonies induced by standard serum pool after mixture with 0.1 ml undialyzed serum). Lines indicate upper limit of range of CSF levels in normal sera and lower limit of range of inhibitor levels in normal sera. Values for normal sera should lie in upper left quadrant. Number of leukemic sera in various categories indicated.

(63%) of the sera, inhibitor levels were subnormal or undetectable. In individual sera, no correlation was observed between CSF and inhibitor levels.

Inhibitor Levels in Control Sera

As controls for the above leukemic sera, assays were performed on inhibitor levels in 106 serum samples from patients with advanced nonhematological malignancies (Table 2). Eighty-two were from patients without infections at the time of sampling and only two had low inhibitor levels. In contrast, 11 of 24 sera from patients with concurrent infections had low inhibitor levels. However, these 11 sera with low inhibitor levels were from two patients (carcinoma of the esophagus and carcinoma of the uterus) and inhibitor levels were in the range of 20 to 45%. Inhibitor levels were also estimated in 34 additional sera from hospital patients with miscellaneous surgical and medical illnesses. Only two sera had subnormal inhibitor levels and these were in a group of 12 taken from patients with secondary infections at the time of sampling. None of the sera from control patients in which inhibitor levels were subnormal exhibited the total lack of inhibitory activity seen in 65 of the 245 leukemic sera.

Serum CSF Versus Urine CSF Output

In the overall survey (Table 1), 751/1422 (53%) of urine samples assayed indicated a daily excretion of CSF above the upper normal limit. A more detailed analysis was made of 185 of these urine samples for which matching serum samples had been obtained during the 24-hr urine collection period. A comparison of the data from these matched specimens is shown in Fig. 3. CSF levels were elevated in 104/185 (56%) of the urines and in 55/185 (30%) of the sera, indicating that these paired specimens were representative of the whole material studied in the survey. Individual paired values varied widely and urine CSF levels were elevated in about half the specimens from patients.
with normal serum CSF levels. However, in patients with high serum CSF levels, a significantly higher proportion 40/55 (73%) also had elevated urine CSF levels ($\chi^2 = 7.7, p < 0.01$), indicating a correlation between high serum CSF levels and high urine CSF output.

**Correlations with Clinical Status**

Six of the patients were still alive when the present analysis was completed but one interesting correlation was apparent between the general assay data and the duration of the disease from diagnosis to death (Table 1). Although no relationship was found between serum or urine CSF levels and disease duration, subnormal serum inhibitor levels were significantly more frequent in patients surviving less than 6 mo (52/67, 78%) than in patients surviving for longer periods (91/182, 50%; $\chi^2 = 14.1, p < 0.01$).

Detailed clinical information was available for patients providing 217 of the sera assayed for CSF and 215 of the sera assayed for inhibitor levels. In this preliminary analysis, patients were classified only for the presence or absence of fever and by general hematological status. Serum CSF levels were elevated more often in patients in partial or complete remission than in patients in relapse (Table 3), although the difference was of only marginal statistical significance. This difference between the two types of patient was due to their differing response to infections. Elevated serum CSF levels were significantly more frequent in patients with fever who were in remission (83%) than in patients with fever in relapse (31%). A rise in serum CSF levels is a normal response to infections$^{14,13}$ and it is clear from the present data that leukemic

**Table 2.—Serum Inhibitor Levels in Control Nonleukemic Patients**

<table>
<thead>
<tr>
<th>Serum Inhibitor Levels</th>
<th>Nonhematological Malignancies</th>
<th>Miscellaneous Surgical and Medical Illnesses</th>
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<td>Subnormal</td>
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<tr>
<td>Total</td>
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<td>24</td>
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</table>

**Fig. 3.—Comparison of serum levels of CSF and urine content of CSF (colonies/0.15 ml standard urine volume) in 185 paired specimens from patients with acute leukemia. Lines indicate upper limit of range of CSF levels in normal serum and urine. All normal values should be in lower left quadrant. Number of paired leukemic specimens in different categories are indicated.**
CSF AND INHIBITORS IN LEUKEMIA

Table 3.—Incidence of Sera With Elevated Colony-stimulating Activity in Relation to Clinical Status

<table>
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<tr>
<th></th>
<th>Fever</th>
<th>No Fever</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Relapse</td>
<td>13/42* (31%)</td>
<td>23/104 (22%)</td>
<td>35/146 (25%)</td>
</tr>
<tr>
<td>Partial or complete remission</td>
<td>15/18 (83%)</td>
<td>15/53 (28%)</td>
<td>30/71 (42%)</td>
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</table>

Significance of difference †

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* Number of sera with high CSF levels/total tested.
† x² test.

patients in relapse tended to be unable to respond to infections by developing elevated serum CSF levels. Pathogenic organisms encountered in the present leukemic patients that elevated CSF levels included *Escherichia coli*, *Staphylococcus pyogenes*, and *Candida albicans*, and the most common types of infection were septicemia, local urinary tract or respiratory infections, and abscesses in soft tissues. In general, the more extensive the infection, the more likely it was for CSF levels to be elevated.

The frequency of subnormal serum inhibitor levels was not significantly different in patients in relapse or remission (Table 4). The development of infections slightly increased the incidence of subnormal inhibitor levels in both types of patient but these differences were not significant statistically. One difference in serum inhibitor levels was noted, however, between patients in relapse and remission. In patients with high serum CSF levels but without infections, serum inhibitors were subnormal in a significantly higher proportion of sera from patients in relapse (86%) than in patients in remission (40%) (Table 5). This latter correlation is in agreement with the association between subnormal inhibitor levels and short survival noted above.

There was no overall correlation between daily urine content of CSF and whether the patient was in relapse or remission. Urine output of CSF was more commonly elevated during episodes of infection (29/40 [73%]) than during afebrile periods (59/128 [46%]; p < 0.01). This response of urinary CSF to infections was seen equally frequently in patients in relapse and remission. Thus, although patients in relapse who developed infections tended to be unable to elevate serum CSF levels, the urine data indicated that some increased production of CSF may have occurred in some of these patients.

DISCUSSION

CSF is a serum glycoprotein which is excreted in the urine and has the specific capacity to stimulate in vitro the proliferation of granulocytes and

Table 4.—Incidence of Sera With Subnormal Inhibitor Levels in Relation to Clinical Status

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<tr>
<td>Relapse</td>
<td>27/40* (68%)</td>
<td>68/106 (64%)</td>
<td>95/146 (65%)</td>
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<tr>
<td>Partial or complete remission</td>
<td>12/16 (75%)</td>
<td>28/53 (53%)</td>
<td>40/69 (58%)</td>
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</tbody>
</table>

Significance of difference †

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* Number of sera with subnormal inhibitor levels/total tested.
† x² test.
Table 5.—Incidence of Sera With Subnormal Inhibitor Levels in Patients With High Serum CSF Levels

<table>
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<tr>
<td>Relapse</td>
<td>9/11* (82%)</td>
<td>18/21 (86%)</td>
<td>27/32 (85%)</td>
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<tr>
<td>Partial or complete remission</td>
<td>11/14 (79%)</td>
<td>6/15 (40%)</td>
<td>17/29 (59%)</td>
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<td>Significance of difference †</td>
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<td><em>p &lt; 0.01</em></td>
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* Number of sera with subnormal inhibitor levels/total tested.
† χ² test.

macrophages. This in vitro action of CSF is blocked by lipoprotein inhibitors which are present in large amounts in most normal sera. There is now some evidence to indicate that CSF functions in vivo as a humoral regulator of granulopoiesis and monocyte formation.

Previous studies have shown that serum CSF levels are elevated in conventional and germ-free mice with leukemia and in some patients with leukemia. The present study of patients with acute granulocytic and myelomonocytic leukemia has shown that CSF levels were elevated in approximately 30% of the sera assayed. Earlier studies suggested that CSF was not detectable in normal sera but was detectable in undialyzed sera from some patients with acute leukemia. These initial results were due to the presence in undialyzed serum of inhibitors that mask the detection of CSF in normal and some leukemia sera. The present data also indicated that abnormally large amounts of CSF were present in about half of the urines examined from patients with acute leukemia.

Acute granulocytic leukemia is a disease characterized by gross abnormalities in granulocytic proliferation and differentiation. These abnormalities may be due in part to abnormal levels of humoral regulators and, although CSF may not be the only regulator controlling granulopoiesis, it is clearly of importance to establish the proliferative “load” placed by CSF on normal and leukemic granulocytes in patients with acute leukemia. In this context, the neoplastic cells of a myelomonocytic leukemia in mice have been shown to be responsive to proliferative stimulation by CSF.

Caution needs to be exercised in interpreting the significance of elevated serum CSF levels. For a classical hormone, serum levels of the hormone probably represent a valid index of the concentration of hormone impinging on the target cells. For CSF, the situation is potentially more complex since some cells in the bone marrow have been shown to produce CSF. Since it has not yet been established whether the bone marrow compartment is a net importer or exporter of CSF from or to the serum, the levels of CSF in the serum need not necessarily give an accurate measure of CSF concentration at the surface of target granulopoietic cells in the marrow. From the present data it is clear also that the level of CSF excretion in the urine does not necessarily give a reliable index of serum CSF levels. However, clearance in the urine appears to be a major metabolic fate of CSF and the high output of CSF in leukemic urine does suggest a higher overall level of CSF production in such patients. Further studies will be required to determine whether such an overall
increase in CSF production places a greater proliferative stimulus on target bone marrow cells, regardless of whether or not serum CSF levels are elevated.

In this initial general analysis of the data, no overall correlation was observed between CSF levels and the hematological status of the patients. In a subsequent paper, detailed data will be presented from sequential studies in individual patients, correlating CSF levels with changes in white cell levels and bone marrow cytology. Certain forms of chemotherapy, e.g., cytosine arabinoside, were found to cause significant changes in CSF levels and these data will also be presented in full in a subsequent paper.

Patients in relapse who developed infections appeared less able than patients in remission to respond by developing high serum CSF levels. In infectious mononucleosis, an inability to develop high serum CSF levels early in the disease has been correlated with a prolonged and difficult clinical course of the disease. It may be that the inability of patients in relapse to develop high serum CSF levels in response to infections is responsible in part for their characteristic susceptibility to infections.

The observation that serum inhibitors of CSF were abnormally low or undetectable in 57% of sera tested is intriguing but the significance of this observation cannot be assessed until evidence is obtained whether these inhibitors have a function in vivo comparable with their action in vitro. It is noteworthy, however, that subnormal inhibitor levels were more common in patients with a short survival time and in certain types of patients in relapse.

Further analyses are required to establish the role of CSF and serum inhibitors in the development and progression of acute leukemia. However, the present data provide clear evidence that abnormal levels of both factors are often present in acute leukemia and support the possibility that many of the features of acute leukemia may not be due solely to intrinsic abnormalities in the affected cells but be due partly to abnormal levels of regulatory factors.

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152


Colony-stimulating Factor and Inhibitor Levels in Acute Granulocytic Leukemia

DONALD METCALF, SOH HA CHAN, FREDERICK W. GUNZ, PAUL VINCENT and ROBERT B. M. RAVICH