Marrow Adherent Cell Colony-stimulating Activity Production in Acute Myeloid Leukemia

By Peter L. Greenberg, Beth Mara, and Philip Heller

Provision of colony-stimulating activity (CSA) by human marrow cells was determined utilizing culture techniques in vitro to assess intramedullary cellular interactions on human granulopoiesis. CSA production from marrow cells of 21 patients with acute myeloid leukemia (AML) was evaluated and compared to normal marrow by testing the capacity of conditioned media from adherent marrow cells to promote granulocytic colony formation in agar of relatively-light-density non-adherent human marrow target cells. Morphologic, cytochemical, density, and phagocytic characteristics of normal marrow cells suggested that marrow CSA production was provided by middensity adherent cells including those of the monocyte-macrophage series. Decreased marrow cell CSA provision was found in 13 of 21 studies of patients with AML at diagnosis or relapse ($p < 0.001$). Chemotherapy induced complete remission in 7 of 8 patients with normal marrow CSA, whereas this was achieved in only 4 of 13 patients with low marrow CSA. In contrast, 42 of 46 studies in 9 patients during complete remission showed normal or increased marrow CSA. Sequential studies in 3 patients with relatively prolonged remissions had repeated normal or increased marrow CSA, whereas intermittent low and decreasing marrow CSA was found in 6 patients with short remissions. These findings suggest that adequate marrow CSA provision may be essential for sustaining normal granulopoiesis in AML and may reflect persistence of a normal monocyte-macrophage cell population. Monitoring this parameter appears useful for evaluating microenvironmental influences on granulopoiesis and assessing prognosis and clinical status in AML.

Investigations in experimental animals have shown that microenvironmental influences within bone marrow and spleen are critical for hematopoietic stem cell proliferation and differentiation.$^{1,6}$ Stromal damage of these organs causes defective hematopoietic cell regeneration.$^{6,8}$ Although the nature of the stromal influences necessary to support hemopoiesis has not been established, histologic and functional studies have indicated that these influences are due in part to locally acting cell-derived factors.$^{1,3,4,6,9}$

Development of marrow culture techniques in vitro has permitted analysis of factors involved in the regulation of granulopoiesis. These studies have shown that granulocytic progenitor cells (GPC) proliferate and differentiate in vitro to form granulocytic-macrophagic colonies in agar (i.e., are colony-forming cells, CFU-C) under the necessary influence of the humoral stimulatory...
substance termed colony-stimulating activity (CSA). CSA is produced by a variety of tissues, with the major cellular sources of CSA in peripheral blood being monocytes (and their progeny, macrophages) and a subpopulation of lymphocytes. Human marrow cells are dependent on cellular sources of CSA for their proliferation in vitro, whereas murine marrow is stimulated as well by CSA present in serum and urine.

Although evidence is accumulating regarding the relevance in vivo of the indices in vitro of granulopoiesis, it should be emphasized that the physiologic role of these parameters has not yet been rigorously established. The relative contributions of the different sources of CSA for granulopoiesis are not known; however, recent studies in mice have shown that marrow GPC proliferation is associated predominantly with intramedullary elaboration of CSA. A close temporal relationship has been shown in vivo between fluctuations in the capacity of marrow cells firmly adherent to the inner surface of hemopoietic bone to produce CSA and the GPC regeneration noted after irradiation. Changes did not occur in CSA production by other organs or in serum CSA levels following low doses of irradiation. These findings are in contrast to the occurrence of elevated serum and extramedullary organ CSA values subsequent to high levels of irradiation or antigenic challenge. These observations suggest that local production of CSA within the marrow may be a major factor in determining the concentration of CSA impinging on target GPC and influencing granulopoiesis.

In man, intramedullary cellular sources of CSA are also present and co-exist with other marrow cells obtained by marrow aspirate. The CSA-producing cells can be preferentially removed from the remaining marrow cell population by their properties of adherence to plastic or glass surfaces and their relatively light buoyant density. Studies in man have suggested a defective capacity of marrow adherent cells to produce CSA in some patients with leukemia and certain neutropenic disorders. Abnormal serum, urine, and peripheral leukocyte CSA levels occur in acute myeloid leukemia (AML). We have utilized adherence and density separation techniques to provide a method to detect, quantitate, and characterize human marrow CSA-producing cells. In order to evaluate the possible role of marrow cell CSA provision as a microenvironmental stimulatory influence on granulopoiesis and to determine its relevance for assessing prognosis and clinical status in granulopoietic disorders, we evaluated marrow cell-derived CSA levels in patients with AML.

MATERIALS AND METHODS

**Marrow colony-forming capacity.** Our techniques of performing assays for granulocytic colony-forming capacity by marrow CFU-C have previously been described. Human marrow cells obtained by aspiration were heparinized, sedimented in 3% dextran, and washed, and 2 x 10^5 cells were plated in a 1-ml 0.3% agar-medium [modified McCoy's 5A medium with 15% fetal calf serum (FCS)] layer over a human leukocyte feeder layer in 1 ml of 0.5% agar-medium. The plates were allowed to gel and were incubated at 37°C in a humidified air-CO2 incubator for 7-10 days. Colonies consisted of groups of >50 cells and were morphologically identified as granulocytic and monocytic cells at various stages of differentiation. Normal CFU-C values were 26 ± 7.5 colonies/10^5 marrow cells plated (mean ± SD).

**Leukocyte-conditioned medium.** A standard soluble CSA source capable of consistent stimulation of human marrow cells was provided by buoyant ( < 1.077 g/cm^3) Hypaque-Ficoll-separated...
human peripheral blood mononuclear leukocytes\textsuperscript{28} incubated at 37\degree C in medium containing
0.5 mM 2-mercaptoethanol at a concentration of 1.2 \times 10^6 cells/ml for 7 days.\textsuperscript{29} The supernatant conditioned medium was harvested after centrifugation, filtered, and stored at \textasciitilde -20\degree C until use. The CSA in batches of leukocyte-conditioned medium has been shown to be stable for
storage periods of at least 1 yr. Conditioned medium (0.15 ml and aqueous dilutions) was used in
single-layer agar culture with marrow target cells, and colony formation was assessed.

\textbf{Marrow cell CSA provision.} Adherent marrow cells were obtained by modification of a pre-
variously described technique.\textsuperscript{22} Marrow cells, diluted in saline, were centrifuged on a Hypaque-
Ficoll gradient (density 1.077 g/cm\textsuperscript{3}) for 20 min at 800 g. The buoyant cells were washed in
McCoy's medium, and 10\textsuperscript{7} cells in 2 ml medium containing 7.5\% FCS were placed in 35 x 10-mm
tissue culture dishes (Falcon Plastics, Oxnard, Calif.) for 30-60 min at 37\degree C. This FCS concentra-
tion improved adherence of CSA-producing cells in comparison with 15\% FCS. Nonadher-
ent cells were removed by rinsing, and the (buoyant) adherent cells were incubated in 1 ml
McCoy's medium with 15\% FCS and 0.5 mM 2-mercaptoethanol for 7 days at 37\degree C in an air-
CO\textsubscript{2} incubator. Nonadherent cells did not produce CSA. Incorporation
of mercaptoethanol into
the medium increased the CSA potency of the conditioned medium approximately sevenfold. Pilot
experiments indicated that 95\% of the CSA was provided by 7 days of incubation. After incuba-
tion the supernatant conditioned medium was harvested and stored at \textasciitilde -20\degree C until use.

The conditioned media were tested for CSA by determining colony formation in single agar
dish culture 7-10 days after plating 0.15-mI serial aqueous dilutions of the media with 0.2 \times
10\textsuperscript{7} buoyant nonadherent normal human marrow target cells. These buoyant cells were obtained by
the neutral-density (density cut) centrifugation procedure described below using an albumin
density at 4\degree C of 1.068 g/cm\textsuperscript{3}. The buoyant (light-density) cells were recovered and per-
mitted to adhere to tissue culture dishes as described above, and the nonadherent cells were re-
covered for use as target cells. Removal of the relatively dense cells from the target marrow
population increased the sensitivity of the system for assessing marrow CSA levels. Control plates
containing nonadherent buoyant marrow target cells without a CSA source were included in all
experiments and consistently showed no colony formation. The number of adherent cells were
calculated by determining the difference between the total number of cells plated and the non-
adherent cells recovered after washing the plates. Direct counting of the adherent cells in mea-
sured areas of the tissue culture dishes generally confirmed the calculated adherent cell values
in representative experiments.

The CSA in test conditioned mediums were compared to that present in the stable human mono-
nuclear leukocyte-conditioned medium CSA standard. Dilution curves of CSA (colonies formed)
permitted quantitative estimates of effective CSA concentrations (termed \(\alpha\)) and were analyzed
by previously described curve-fitting computer programs.\textsuperscript{30} All specimens (0.15 ml) were tested
undiluted and in serial aqueous dilutions of 0.5x, 0.25x, and 0.063x. The following equation
was used to determine CSA levels in the conditioned media:

\[
\text{CSA (U/ml)} = 6.66 \times \alpha^{x} \times l_{x} / \alpha_{s},
\]

where a unit of CSA is defined as the amount necessary for stimulating one colony in vitro,
\(\alpha_{s}\) and \(\alpha_{t}\) represent the computer-calculated effective CSA concentrations of the unknown
test medium and the standard leukocyte-conditioned medium, respectively, and \(l_{x}\) represents
the number of colonies stimulated by the undiluted 0.15-mI leukocyte-conditioned medium.
Multiplication by 6.66 converts CSA from 0.15 to 1 ml.

\textbf{Albumin density separation procedures.} Our procedures for performing the bovine serum albu-
min continuous gradient equilibrium centrifugation technique and the neutral-density (density
cut) procedure for evaluating density distribution patterns of marrow cells have previously been
described.\textsuperscript{27,28} For continuous gradients, linear albumin gradients were generated in the density
range 1.055-1.075 g/cm\textsuperscript{3} and contained 10\textsuperscript{7}-10\textsuperscript{9} marrow cells. Gradients were centrifuged for
40 min at 5000 g at 4\degree C. Then 9-14 fractions were collected at 4\degree C by upward displacement
and centrifuged after dilution with McCoy's medium; the pellet was then resuspended in medium
and plated for CFU-C or CSA determination. For determining cellular CSA provision the frac-
tions were permitted to adhere to plastic tissue culture dishes after density separation; condi-
tioned medium was then produced and harvested as described above. Refractive index (Bausch
and Lomb Abbe-3L Refractometer) was routinely employed for evaluating fraction density, since
this parameter correlated well with direct density measurements. Continuous gradient density

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distribution profiles of CFU-C were calculated as CFU-C per density increment against fraction density. For the neutral-density procedures, the bone marrow cells were dispersed in albumin (density 1.068 g/cm³) and centrifuged for 10 min at 4000 g at 4°C. Cells in suspension and pellet were recovered separately and diluted in medium, centrifuged, resuspended, and plated in agar for determination of CFU-C and CSA.

**Morphology and cytochemistry.** The morphology of the adherent cells was determined by permitting cells to adhere to glass cover slips in tissue culture dishes. The cover slips were then washed with medium to remove loosely adherent cells, stained with Wright-Giemsa or esterase stains, and examined microscopically (100-300 cell differential counts). The method we used to perform the α-naphthyl acetate esterase stain for identification of monocytes has previously been described.32

**Phagocytic activity.** Adherent cells in 1 ml complete medium on glass cover slips were exposed to 20 λ of polyvinyltoluene latex particles (2 μm, Dow Chemical, Indianapolis, Ind.) for 1 hr at 37°C in an air-CO₂ incubator.33 The medium was removed, the cells were washed to remove noningested particles, and the cover slips were fixed and stained with Wright-Giemsa stain. Uningested latex particles adhering to the surface of the mononuclear cells were removed by exposure to xylene as previously described.34 This xylene exposure did not diminish the number of intracellular particles. The proportion and morphology of adherent cells ingesting latex particles were determined.

**Patients.** Bone marrow aspirates for assessment of control marrow cellular CSA provision and for target cells for the test conditioned media were obtained from normal volunteers or from patients whose marrows had been obtained at time of surgery for nonhematologic disorders. Marrow aspirates were performed after informed consent was obtained according to guidelines established by the Stanford University Human Experimentation Committee. These individuals had normal complete blood counts and marrow morphology.

Twenty-one patients with AML at various stages of diseases were studied. Fifteen had acute myeloblastic leukemia (AMyL) and six had acute myelomonocytic leukemia (AMML). Morphologic distinctions between myeloblastic and myelomonocytic leukemia were based on previously described criteria.35 It is recognized that this morphologic categorization of cell type is less sensitive and specific than current cytochemical criteria.36 Marrow categories of clinical status in AML were described as previously defined: M3 indicated marrow containing >25% blasts, M2 5%-25% blasts, and M1 <5% blasts and normal hemopoiesis, i.e., complete remission.37

Chemotherapy for patients with AML at diagnosis or relapse consisted of intensive courses of daunorubicin, cytosine arabinoside (ara-C), and 6-thioguanine.38 Patients in remission received monthly 2-day pulses of ara-C and 6-thioguanine for maintenance therapy as previously described.39 Marrow aspirates in all patients were performed prior to courses of chemotherapy. The maintenance regimen utilized had been shown to be relatively nontoxic, with return to normal granulopoietic parameters by 1 mo after therapy.40 The ages of patients with M3 marrows in our present study were 19-81 yr (median 56 yr).

Statistical analyses were performed by the Wilcoxon two-sample two-tailed rank test and the χ² test with Yates correction. Differences were considered statistically significant if p < 0.05.

**RESULTS**

**Marrow cell CSA provision.** Sigmoid-shaped dose-response curves of CSA production relative to the number of adherent cells per plate were found, with a plateau occurring with approximately 3-15 × 10⁵ adherent cells (Fig. 1). In normal individuals, the number of adherent cells generally paralleled the number of marrow cells plated. Marrow CSA provision from 16 control subjects was 34.4 ± 26.1 U/ml (mean ± SD). The mean number of adherent cells per dish after plating 10³ marrow cells was 8.6 ± 3.2 × 10⁵, i.e., 8.6%, with a range of 1.5-25 × 10⁵ cells.

We evaluated the contribution to marrow CSA production by nonadherent and adherent cells. As shown in Table 1 all of the CSA was provided by the adherent cells. Concentrations of nonadherent cells used as target cells for test
samples, and much higher concentrations, were shown not to produce CSA. The necessity of depleting marrow of adherent cells in order to assess CSA from various sources, including plasma, was further assessed. As shown in Table 2, endogenous CSA-producing cells were present in populations in which adherent cells had not been removed. No CSA was detected in plasma samples (including serial dilutions) when nonadherent marrow cells were utilized as target cells, whereas enhancement of colony formation by endogenous CSA-producing cells was found when unseparated marrow target cells were used.

**Characterization of marrow CSA-producing cells.** Characterization of the marrow cells that produced CSA was performed by evaluating the morphology, phagocytic activity, α-naphthyl esterase positivity, and density distribution profiles of the adherent cells: 86.9% ± 5.2% of the adherent marrow cells

### Table 1. CSA Production by Adherent and Nonadherent Marrow Cells

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Adherent Cells*</th>
<th>Nonadherent Cells*</th>
<th>Nonadherent Buoyant Cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Cells (x 10^5)</td>
<td>Colonies</td>
<td>No. Cells (x 10^5)</td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>57 ± 5</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>77 ± 6</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>65 ± 2</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>85 ± 3</td>
<td>72</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Conditioned media were prepared by incubating adherent or nonadherent cells in nutrient medium for 7 days.
†Cells were obtained by depleting buoyant (<1.068 g/cm²) cells of adherent cells prior to preparing conditioned medium.
‡Colonies per 0.2 x 10^6 nonadherent buoyant (<1.068 g/cm³) normal human marrow target cells stimulated by 0.15-ml conditioned media. Positive (leukocyte-conditioned medium) and negative (saline) controls stimulated 57 ± 4 and 0 colonies, respectively.
from control subjects appeared to be monocytes by Wright-Giesma stain, 85% ± 3.8% were esterase positive, and 84% ± 7.6% ingested latex particles. Continuous density gradient separation of the adherent cells indicated that peak CSA occurred at a density of 1.066 g/cm³ (Fig. 2) and was not related to adherent cell number. As shown, this peak was more dense than the peak incidence of CFU-C.

Further characterization of the CSA-producing cells was performed by determining the morphology, α-naphthyl acetate esterase positivity, and phagocytic activity of continuous density gradient separated adherent marrow cells (three separate experiments). As shown in Fig. 2 the cells providing CSA (density region 1.064–1.072 g/cm³) were comprised of 60%–84% monocytes, 87%–94% esterase-positive cells, and 60%–80% phagocytes. The lower propor-
tion of relatively dense esterase-positive cells was due to a decreased percentage of monocytes (higher percentage of neutrophils) in these fractions. In the very light fractions these monocyte markers were present despite low CSA provision. In the middensity region an increased proportion (24%–39%) of lymphocytes was found.

In order to determine the possible contribution of peripheral blood CSA production to adherent marrow cell CSA levels, density separation of these cell populations was performed. As shown in Fig. 3, describing a representative pilot experiment, marrow and peripheral blood adherent CSA-producing cells had differing density distribution profiles. Peripheral blood peak adherent CSA-producing cells were more dense than those of marrow. Similar numbers of adherent cells were present per fraction for the two sources and were adequate to give plateau level CSA production. However, as shown, adherent peripheral blood cells provided much lower amounts of CSA (9% of the marrow values in peak fractions). The percentage of adherent cells from unfraccionated peripheral blood was found to be similar to that in marrow, 8.4% ± 1% (mean ± SE). Mixing experiments were performed, and at a 1:1 ratio of marrow and peripheral blood no additional CSA was provided by the peripheral blood cells.

To further determine the functional relationship between human marrow and peripheral leukocyte CSA, these cellular CSA sources were tested for their ability to stimulate colony formation by CFU-C of different densities. As indicated in Fig. 4, conditioned media from adherent marrow cells and peripheral mononuclear leukocytes and leukocyte feeder layers showed peak stimulatory activity for marrow CFU-C of the same density.

**Marrow CSA in AML.** In four experiments with marrow cells from AMyL patients with M3 marrows, 7.5% ± 4.5% (mean ± SE) of the cellular CSA was provided by nonadherent or dense cells in comparison with that provided by the adherent cell populations. In order to optimize detection of CSA production from these patients we routinely assessed this parameter by analyzing adherent cell populations. Table 3 describes the clinical data and data in vitro obtained from patients with AML at diagnosis or relapse. Figure 5 shows composite values of adherent marrow cell CSA provision from patients with AML.
MARROW CELL CSA PRODUCTION IN AML

Fig. 4. Density distribution profile of marrow colony-forming cells stimulated by CSA derived from marrow and peripheral leukocyte sources.

at various stages of disease; 21 determinations were performed in 17 patients at the time of diagnosis or relapse (M3 marrows). As indicated, low marrow CSA values were found in 13 of 21 determinations in patients with M3 marrows. Low CSA values were considered to be those beyond 1 SD of the control values. Marrow CSA values from AML patients were significantly lower than those of control subjects, particularly for those patients failing to enter chemotherapy-induced complete remission (Table 4). Similarly, only 4 of 13 patients (31%) with low marrow CSA values entered complete remission, whereas 7 of 8 patients (88%) with normal marrow CSA did so ($p < 0.05$). The overall complete remission rate for patients in this study was 52% (11/21) patients. Data from all AML patients with M3 marrows were analyzed together, whether they were previously untreated, relapsed, AML, or AMML, since their therapy and complete remission rates were essentially the same.

Fig. 5. Adherent marrow cell CSA values of normal subjects and patients with acute myeloid leukemia. M1, complete remission; M2, partial remission; M3, relapse or diagnosis. ●, M3 patients who achieved complete remission. Stippled area, mean ± SD of control CSA values.
Table 3. Clinical Data and Culture Data In Vitro of Patients With AML at Diagnosis or Relapse

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)/Sex</th>
<th>Diagnosis*</th>
<th>Peripheral Blood</th>
<th>Marrow</th>
<th>Marrow Studies In Vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hct (%)</td>
<td>Platelets (x 10^12/mm^3)</td>
<td>WBC (x 10^3/mm^3)</td>
<td>Blasts (%)</td>
<td>Response</td>
</tr>
<tr>
<td>1</td>
<td>43/F</td>
<td>AMMLD</td>
<td>31</td>
<td>165</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td>56/M</td>
<td>AMylD</td>
<td>32</td>
<td>14</td>
<td>3.3</td>
</tr>
<tr>
<td>3</td>
<td>58/M</td>
<td>AMMLD</td>
<td>15</td>
<td>20</td>
<td>50</td>
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<tr>
<td>4</td>
<td>38/F</td>
<td>AMylA</td>
<td>37</td>
<td>144</td>
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<td>5-1</td>
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<td>46/M</td>
<td>AMylA</td>
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<td>18</td>
<td>75</td>
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<tr>
<td>6-1</td>
<td>58/M</td>
<td>AMylD</td>
<td>32</td>
<td>58</td>
<td>2</td>
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<tr>
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<td>58/M</td>
<td>AMylD</td>
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<td>155</td>
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<td>AMMLD</td>
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<td>66/M</td>
<td>AMMLD</td>
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<td>8</td>
<td>46/F</td>
<td>AMylD</td>
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<td>10</td>
<td>1.5</td>
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<tr>
<td>9</td>
<td>62/F</td>
<td>AMylD</td>
<td>35</td>
<td>19</td>
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<tr>
<td>10</td>
<td>68/F</td>
<td>AMylD</td>
<td>24</td>
<td>121</td>
<td>172</td>
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<td>11</td>
<td>19/M</td>
<td>AMylA</td>
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<td>AMylA</td>
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<td>17</td>
<td>20/F</td>
<td>AMMLD</td>
<td>38</td>
<td>44</td>
<td>117</td>
</tr>
</tbody>
</table>

Normal values: 8.6 ± 3.2, 34.4 ± 26.1, 26 ± 7.5

*AMML, acute myelomonocytic leukemia; AMyl, acute myeloblastic leukemia; subscript D indicates patient studied at diagnosis; subscript R denotes patient studied at relapse. CR, complete remission achieved; F, failure of complete remission induction.

1 Serial dilutions of adherent cell-conditioned media were used to stimulate 0.2 x 10^5 nonadherent buoyant (<1.068 g/cm^3) marrow target cells; 0.15-ml stimuli used in single agar layer culture.

1 Myeloid colonies formed upon stimulation with human leukocyte feeder layers as CSA source in double agar layer culture.

§Cluster formation noted in these cultures.
Table 4. Adherent Marrow Cell CSA Production in AML

<table>
<thead>
<tr>
<th>Adherent Marrow Cell Source</th>
<th>Number of Determinations</th>
<th>CSA1 (U/ml)</th>
<th>p $^\dagger$</th>
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</thead>
<tbody>
<tr>
<td>Normal Controls</td>
<td>16</td>
<td>34.4 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukemia (AML)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnosis/relapse (M3) — all patients</td>
<td>21</td>
<td>20.1 ± 7.3</td>
<td>0.01$^\ddagger$</td>
</tr>
<tr>
<td>Diagnosis/relapse patients achieving CR</td>
<td>11</td>
<td>29.4 ± 11.8</td>
<td>NS</td>
</tr>
<tr>
<td>Diagnosis/relapse patients failing CR</td>
<td>10</td>
<td>9.9 ± 7.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Diagnosis/relapse AMyL$^\ddagger$</td>
<td>14</td>
<td>9.4 ± 3.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Diagnosis/relapse AMML</td>
<td>7</td>
<td>41.3 ± 19.2</td>
<td>NS</td>
</tr>
<tr>
<td>Partial remission (M2)</td>
<td>4</td>
<td>34.2 ± 8.6</td>
<td>NS</td>
</tr>
<tr>
<td>Complete remission (M1)</td>
<td>46</td>
<td>51.2 ± 7.9</td>
<td>NS</td>
</tr>
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</table>

$^*10^7$ marrow cells plated per dish providing adherent marrow cells incubated in nutrient medium for 7 days.
10.15 ml of serial dilutions of conditioned media were used to stimulate 0.2 $\times$ $10^5$ nonadherent buoyant (< 1.066 g/cm$^3$) normal human marrow target cells; mean ± SE.

$^\dagger$ M1, marrow morphology consisted of 0%-5% myeloblasts with normal hemopoiesis (i.e., complete remission, CR); M2, 5%-25% myeloblasts; M3, >25% myeloblasts.

$^\ddagger$ Numbers in parenthesis indicate p values of statistical differences between patients’ CSA levels and those of control subjects. NS, not significantly different.

AMyL, acute myeloblastic leukemia; AMML, acute myelomonocytic leukemia.

(54%, 52%, 50%, 57%, respectively). Four determinations in two patients in partial remission (M2) and 42 of 46 determinations in ten patients during complete remission (M1 marrows) were normal (Table 3, Fig. 5).

CSA levels relative to the number of adherent cells (rather than to the total cells plated, i.e., $10^7$ cells plated) was also evaluated to assess possible qualitative as well as quantitative decreases in CSA production. Absent marrow CSA provision was associated with $\leq 1\%$ adherent marrow cells, i.e., $\leq 1 \times 10^5$ adherent cells ($p < 0.01$). Six of seven patients with M3 marrows and absent CSA had $\leq 1\%$ adherent cells, and six of eight patients with $<1\%$ adherent cells lacked CSA provision. The 13 patients with $>1\%$ adherent cells had no significant decrease of CSA provision, indicating lack of qualitative defect in CSA production by these adherent cells. Achievement of complete remission was not significantly associated with the percentage of adherent cells.

In order to determine whether low CSA values found in several patients with M3 marrows were related to the presence of inhibitory substances in the conditioned media directed against CFU-C or to lack of CSA production, experiments were performed in which varying proportions of leukemic adherent cell-conditioned media lacking CSA were mixed with an active leukocyte-conditioned medium CSA source. In the four studies performed no evidence of inhibition was found when tested against nonadherent buoyant normal human marrow cells. Experiments were also performed to determine if the lack of CSA provision was related to dilution of CSA-producing cells by the high percentage of blasts present. Density separation was performed in two patients to remove a high proportion of the relatively-light-density blasts, and conditioned media from both cell fractions (< and >1.066 g/cm$^3$) were used as a source of CSA. The low CSA values persisted despite adequate numbers of adherent cells in each cell fraction to give plateau CSA values. The morphology of these adherent cells was not assessed.
The relationship between clinical data and data in vitro in Table 3 was further evaluated. No correlation was found between the patients' ages, peripheral leukocyte counts, percentage or absolute numbers of blasts, untreated or relapsed status, and the incidence of complete remission or marrow CSA values. Although slightly higher mean marrow blast percentages were found in AML patients with low versus normal marrow CSA (80.6% ± 5.5% versus 70.1% ± 8%), (mean ± SE), ≤1% versus >1% adherent marrow cells (85.7% ± 4.7% versus 71.0% ± 6.5%), and failures versus patients achieving complete remission (79.3% ± 7% versus 74.2% ± 6.3%), the differences present in these patient groups were not statistically significant. Adherent cell morphology in patients with M3 marrows showed a mixture of blasts and monocytes. AML subjects with either normal or low marrow CSA had a significantly decreased proportion of monocytes in the adherent cell population (56.8% ± 10.6% and 13% ± 7.4%, respectively, p < 0.05) in comparison with control subjects. The relatively low adherent monocyte values in AML patients with normal versus low CSA were not significantly different from each other.

Of 17 patients, 6 (7 of 21 determinations) having marrow CSA evaluation at the time of diagnosis or relapse had AMML; the remainder had AMyL. Although higher mean marrow CSA values were found in AMML, the levels were not significantly different. Marrow CSA levels were significantly lower in AMyL patients than in controls (Table 4). There was no significant difference in the percentage of adherent cells in patients with AMML compared to AMyL. Achievement of complete remission was similar in both groups (4 of 7, 57% versus 7 of 14, 50%).

Sequential studies of marrow CSA were evaluated in nine patients with AML in remission. Three of these patients were in stable remission for at least 10 mo (Fig. 6) and persisted in remission for longer than 8 mo since their last marrow CSA determination. As shown, in these patients normal sequential CSA values were found. Six patients followed while in remission subsequently relapsed and had intermittent low or progressive decreases of marrow CSA within 2-3 mo prior to relapse (Fig. 7).

**Marrow CFU-C in AML.** We evaluated marrow CFU-C incidence to determine if this value correlated with marrow CSA values or prognosis in AML.
Fig. 7. Granulocytic CFU-C and adherent cell CSA values from marrows of patients with AML during remission until relapse.

As shown in Table 3, 19 of 20 CFU-C values were below normal. Three patterns of growth were found—no growth (11 patients), colony formation (9), or cluster formation (3, 2 without and 1 with colony formation). Although patients with no colony formation had a lower incidence of subsequent complete remission than patients with colony formation (36% versus 78%), these differences were not statistically significant. Six of seven marrow CFU-C determinations in patients with AMML showed no growth, whereas this absence of colony formation occurred in only 5 of 13 determinations of marrow from patients with AML. As a group, patients with AMML had significantly lower marrow CFU-C values than patients with AML (p < 0.05).

All 7 AML patients with M3 marrows who had no demonstrable marrow CSA production also lacked marrow CFU-C. Conversely, marrow cells from 4 of 11 (36%) patients with no colony formation did produce CSA. Only 2 of 7 (28%) patients lacking both marrow CFU-C and CSA (or absent CSA only) achieved complete remission. The relationships between marrow CFU-C and CSA during remission are noted in Figs. 6 and 7. As shown, marrow CSA paralleled marrow CFU-C values until impending relapse, when CFU-C decreased before CSA.

DISCUSSION

These studies quantitate human marrow cell CSA provision, characterize the marrow CSA-producing cells, and assess alterations of marrow cell CSA levels in AML. Our data have confirmed and extended prior studies demonstrating that marrow CSA is provided by the adherent cell population. Of critical importance for assessing human marrow CSA provision is the use of human marrow target cells depleted of these endogenous CSA-producing cells. That the adherence procedure accomplished this was indicated by lack of CSA elaboration by the nonadherent target cells. The absence of CSA in plasma from marrow or peripheral blood further implicates local cellular sources as...
being important providers of CSA. Our finding that plasma enhanced CSA production by endogenous human CSA-producing cells rather than provided CSA directly has also recently been duplicated by other workers. Removal of dense marrow cells from the target cell population increased the sensitivity of the assay system, either as previously suggested by diminishing inhibitory influences provided by the dense neutrophils or by CFU-C enrichment.

Our data describing morphologic, cytochemical, phagocytic, and adherence characteristics of human marrow cells are consistent with the thesis that cells of the monocyte-macrophage series contribute to and may provide the majority of marrow CSA (Fig. 2). These cells have been shown to be active CSA sources from other tissues. However, light-density adherent marrow cells with otherwise similar cellular characteristics provide little CSA, suggesting functional heterogeneity of these marrow monocyte-macrophages. Functionally distinct subclasses of density-separated murine macrophages were previously shown. Alternatively, since density separation did not show precise correlation of CSA provision with monocytes, it is possible that another cell type or interacting cells may significantly contribute to marrow CSA production.

Marrows from control subjects had an adequate number of adherent cells to provide CSA levels on the plateau of the dose-response curve of these marrow cells (Fig. 1). Density distribution profiles indicated that the peak of marrow CSA-producing cells represents a subpopulation of the adherent cells and was of a heavier density than the peak of marrow CFU-C (Fig. 2). These findings were also found in prior studies with human marrow and peripheral leukocytes that did not use adherence separation. Human marrow CSA and leukocyte CSA stimulated CFU-C of similar density (Fig. 4), indicating similar marrow target cells for these CSA sources.

The major contribution to adherent marrow CSA levels was provided by endogenous adherent marrow cells, since adherent peripheral leukocytes produced much lower amounts of CSA (Fig. 3) and addition of peripheral leukocytes to marrow cells added no additional CSA. Furthermore, peripheral blood leukocytes and monocytes are quantitatively fewer than the number of these cells present within the marrow, and in our study approximately equal percentages of adherent cells were found in peripheral blood and marrow. Different density profiles of adherent CSA-producing cells were found for marrow and peripheral leukocytes (Fig. 3). The more dense peak of peripheral leukocyte adherent cells producing CSA was similar to that shown previously for peripheral leukocytes unseparated by adherence procedures.

Our studies of adherent marrow cell CSA provision in AML showed that alteration of this parameter correlated well with the patients' clinical status and prognoses. Patients at diagnosis or relapse with M3 marrows had significantly decreased marrow CSA values, particularly patients with AMyL and those failing remission induction (Table 4, Fig. 5). Low marrow CSA was a negative prognostic indicator, since only 4 of 13 (31%) patients with this finding entered drug-induced complete remission, whereas complete remission occurred in 7 of 8 (88%) patients with normal CSA. Other presenting clinical features analyzed for correlation with marrow CSA or achievement of complete remission showed no significant differences.
Defective CSA provision was apparent in some of our AML patients despite adequate numbers of adherent cells. Prior studies have also shown that differential adherence of leukemic versus normal CSA-producing cells does not account for low CSA values\(^2\) and that abnormal CSA release occurs from AML cells.\(^3\) These data suggest a qualitative defect of CSA provision in the adherent marrow cell population of these patients. In AML patients with low CSA values, no evidence of inhibitory substances against CFU-C was found. The conditioned media were incubated for 7 days; thus the recently described labile inhibitory substance against CFU-C elaborated by leukemic cells\(^4\) would not have been detected optimally in our culture system. It has been further shown that leukemic cells generate no inhibitory activity against CSA production.\(^5\) Separation of relatively-light-density blasts from marrow cell population did not enhance CSA production, indicating that low CSA provision in these patients was not related to dilution of CSA-producing cells by the mass of buoyant leukemic blasts.

The marrow CFU-C growth patterns in our patients with AML were similar to those previously described from this laboratory.\(^2\),\(^3\),\(^10\) Although patients with demonstrable colony formation had a higher complete remission rate than those with no growth, this difference was not statistically significant. As a group patients with AMML had significantly lower marrow CFU-C incidence, with 6 of 7 patients having no colony formation. This difference was not found in a prior study of myeloid growth patterns in AML.\(^6\) However, since morphologic subdivision of patients into AMyL and AMML categories is less sensitive and specific than evaluation by cytochemical methods,\(^3\) confirmation of this aspect of the study will need the application of specific cytochemistry. Marrow CFU-C growth patterns have previously been shown to have prognostic value in AML, although differing profiles have been presented by several investigators.\(^6\),\(^7\)

Sequential investigations showed that marrow CSA provision was generally normal in patients during stable remission. In contrast, low or progressively decreasing marrow CSA values occurred in patients with impending relapse. Marrow CFU-C and CSA correlated well in these patients during remission, with a decrease in marrow CFU-C occurring earlier than low marrow CSA in impending relapse. This prolonged persistence of marrow CSA may relate to the relatively longer lifespan of monocyte-macrophages in comparison with GPC.\(^8\)

The findings that normal marrow CSA values were associated with both achievement of complete remission and with persisting remission suggest that adequate marrow CSA provision may be essential for sustaining normal granulopoiesis in AML following drug-induced marrow hypoplasia and during remission. As such, this parameter may measure intramedullary influences involved in regulating granulopoiesis. Recent preliminary studies have suggested that lithium carbonate, a drug that increases CSA production,\(^9\) enhances neutrophil recovery following chemotherapy in AML.\(^10\) We are currently investigating the utility of this approach, particularly for patients in whom low marrow CSA is evident, in a randomized clinical trial. Such studies may aid in ascribing relevance in vivo for the parameters being assessed in vitro. Histologic examination of human marrow has indicated that granulopoiesis
occurs within the marrow parenchyma, where granulocytic precursors and cells of the monocyte-macrophage series are in proximity. Thus it is possible that short-range interaction occurs in this microenvironment between adherent cell-elaborated stimulatory substances and granulocytic precursors. In this model, long-range influences could also contribute in response to major perturbations such as infection or antigenic challenge.

Prior culture and chromosome data in vitro have suggested the coexistence of normal and leukemic cells in marrows of patients with AML. Parallel changes of marrow CFU-C and CSA and the association of monocytes in the adherent cell population suggest that marrow cell-derived CSA provision may reflect persistence of a normal monocyte-macrophage population, especially in AMyL, where low CSA levels are found. Alternatively, certain adherent leukemic cells could produce CSA, particularly, as has been suggested, in patients with AMML. However, in these latter studies coexisting normal and leukemic cells could have been present and endogenous CSA-producing cells not depleted from the target marrow cells could have been the source of the observed CSA. Functional maturation along monocylic lines appeared necessary for CSA elaboration. The difficulty of morphologically distinguishing normal from leukemic monocytic cells has been demonstrated, indicating the need for specific markers depicting cells of normal or leukemic origin to resolve this point.

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Marrow adherent cell colony-stimulating activity production in acute myeloid leukemia

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