Serial In Vitro Marrow Culture in Acute Myelocytic Leukemia


The in vitro granulocyte colony-forming ability of bone marrow from 19 patients with acute myelocytic leukemia (AML) was studied at the time of initial diagnosis and serially. Initially 18 of the 19 patients grew scant numbers of colonies, one produced large numbers of colonies. When clinical remission was attained and leukemic blasts in the marrow were reduced to <5%, colony forming ability usually returned to normal. Eight such patients who achieved clinical remission were serially studied during remission. Normal in vitro colony formation ensued in five of the eight patients studied. Three patients did not produce normal numbers of colonies, and it was noted that these patients relapsed within 2 mo. The repeated observation of normal numbers of granulocyte colonies appears to distinguish patients whose remissions are stable from those patients who relapse quickly.

Granulocytic colony formation in vitro is a measure of granulocyte proliferative capacity in vivo. The initial marrow granulocyte growth is abnormal in patients with acute myelocytic leukemia (AML). While an unusual untreated patient exhibits exuberant colony growth, most investigators note no colony growth or the presence of small clusters containing less than 20 cells per colony in patients with active disease. While scant colony formation is seen in relapse, colony growth can be normal in remission. Prior reports of the in vitro marrow growth of patients with AML have emphasized isolated single observations of granulocyte colony formation. This report documents the poor colony growth at initial diagnosis of 18 of 19 patients with AML. In addition, serial studies of eight patients who achieved clinical remission demonstrate that colony forming ability (CFU-c) correlates with remission duration.

MATERIALS AND METHODS

Marrow Culture Technique

The method of collection of human bone marrow and the methylcellulose tissue culture technique are as previously described. Marrow cultures were done during the 18-mo period from December 1970 through May 1972. All marrow samples were cultured with and without human embryonic kidney cell supernate (HEKS) as colony stimulating factor (CSF). HEK cells (Microbiological Associates, Inc., From the National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.


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Bethesda, Md.) are grown in 10°, CO₂ humidified incubator in loosely capped culture bottles until a confluent monolayer is achieved. The old media is poured off and 50 cc of CMRL 1066 and 20°, fetal calf serum is added to the culture bottle. This supernate is incubated for 7 days in 10°, CO₂ humidified incubator, then decanted off, centrifuged, pooled, and frozen. Although two different lots of HEKS were used, each patient’s serial marrow sample was cultured with the same lot. The serial marrow samples were plated with the same lots of fetal calf serum and bovine serum albumin. A differential count of the marrow aspiration was obtained before processing the sample. After processing the marrow a second differential was done on the sample prior to plating the cells. At no time was there a major change in the percentage of immature cells between the samples.

Normal Controls

Bone marrows from 24 normal individuals were obtained according to a protocol approved by the Medical Board of the National Institutes of Health. The normal healthy controls had peripheral leukocyte counts within the normal range (5000–10,000), with normal white cell differential counts. The marrow was normally cellular on smear, with normal M:E ratios. The control data is discussed in detail in another paper.  

Patient Population and Management

All patients were under the care of the Hematology and Supportive Care Branch of the National Cancer Institute. Nineteen patients were studied at the time of initial diagnosis and prior to therapy. The patients’ ages ranged from 9 to 63 yr. Marrows from eight patients were serially cultured (Table 1). Their ages ranged from 9 to 60 yr. The median time during which serial CFU-c evaluation were obtained was 7 mo from the time of diagnosis, with a range of 3–9 mo.

Patients were treated with two major intensive combination chemotherapy protocols. The first protocol consisted of a combination of cytosine arabinoside (100 mg/sq m intravenously q 12 hr) and 6-thioguanine (90 mg/sq m orally every 12 hr) given until marrow aplasia occurred. The schedule was repeated when normal bone marrow elements recovered. The second protocol included the intensive use of alternate four-drug combinations known respectively as POMP and PRAVD. POMP includes prednisolone (1000 mg/sq m × 5 days), vincristine (2 mg/sq m on day 1), methotrexate 7.5 mg/sq m × 5 days, and 6-mercaptopurine (500 mg/sq m × 5 days). PRAVD includes prednisolone (1000 mg/sq m × 5 days), cytosine arabinoside (100 mg/sq m × 5 days), vincristine (2 mg/sq m on day 1), and daunorubicin (30 mg/sq m on days 4 and 5). These courses were repeated alternately upon recovery of normal bone marrow cellularity.

Bone marrow samples for in vitro culture were obtained just prior to the next sequential course of chemotherapy, usually from 7 to 28 days after the preceding chemotherapy course. A remission marrow was defined as a bone marrow that was normocellular, with representation of all normal cellular elements, and 5°, or less abnormal myeloblasts. It is emphasized that following chemotherapy, leukemic myeloblasts cannot always be distinguished from regenerative normal myeloblasts. Estimations of marrow cellularity were made by examining the clot sections of aspirated bone marrow. Marrows estimated to have less than 30°, of the total area of evaluable particles represented by hematopoietic precursors were classified as hypocellular. Those sections with 70°, or more of their total area occupied by cellular elements were classified as hypercellular.

RESULTS

The data from serial patient studies and from normals are presented in two ways: (1) colonies/10,000 marrow nucleated cells plated with colony-stimulating factor (HEKS), and (2) colonies/10,000 marrow nucleated cells plated without colony-stimulating factor.
Table 1. Hematologic Data on Eight Serially Studied Patients with AML

<table>
<thead>
<tr>
<th>Patient Hospital No.</th>
<th>Age/Sex</th>
<th>Chemotherapy</th>
<th>Initial Marrow Blast (%)</th>
<th>Initial Marrow Cellularity</th>
<th>Initial Peripheral Count/% Mature Granulocytes</th>
<th>Time to First Remission (Days)</th>
<th>Initial Remission Peripheral Count/% Mature Granulocytes</th>
<th>Initial Remission Marrow Blast (%)</th>
<th>Initial Remission Duration (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW 08-96-05</td>
<td>29♂</td>
<td>Ara-C/6TG</td>
<td>90</td>
<td>Hyper</td>
<td>2.400/02%</td>
<td>20</td>
<td>5</td>
<td>2300/2%</td>
<td>270+</td>
</tr>
<tr>
<td>JD 08-96-18</td>
<td>15♀</td>
<td>POMP/PRAVD</td>
<td>90</td>
<td>Hyper</td>
<td>239.000/04%</td>
<td>45</td>
<td>5</td>
<td>2300/38%</td>
<td>256+</td>
</tr>
<tr>
<td>SS 08-91-52</td>
<td>9♀</td>
<td>PRAVD</td>
<td>80</td>
<td>Hyper</td>
<td>9.200/41%</td>
<td>85</td>
<td>3</td>
<td>3500/86%</td>
<td>192</td>
</tr>
<tr>
<td>RR 08-67-35</td>
<td>22♂</td>
<td>POMP/PRAVD</td>
<td>90</td>
<td>Hyper</td>
<td>37.900/19%</td>
<td>83</td>
<td>3</td>
<td>6900/29%</td>
<td>166</td>
</tr>
<tr>
<td>GK 08-82-35</td>
<td>32♀</td>
<td>POMP/PRAVD</td>
<td>49</td>
<td>Hyper</td>
<td>23.400/20%</td>
<td>47</td>
<td>5</td>
<td>2700/34%</td>
<td>263</td>
</tr>
<tr>
<td>FD 08-94-24</td>
<td>60♂</td>
<td>Ara-C/6TG</td>
<td>78</td>
<td>Hyper</td>
<td>78.000/41%</td>
<td>51</td>
<td>2</td>
<td>3200/92%</td>
<td>35</td>
</tr>
<tr>
<td>AK 08-92-54</td>
<td>36♀</td>
<td>Ara-C/6TG</td>
<td>50</td>
<td>Normo</td>
<td>3.600/30%</td>
<td>73</td>
<td>3</td>
<td>1400/15%</td>
<td>52</td>
</tr>
<tr>
<td>EM 08-91-14</td>
<td>58♂</td>
<td>POMP/PRAVD</td>
<td>75</td>
<td>Hyper</td>
<td>16.700/14%</td>
<td>43</td>
<td>&lt;1</td>
<td>8800/87%</td>
<td>47</td>
</tr>
</tbody>
</table>
Table 2. Colonies/10,000 Nucleated Cells Plated With HEKS at Initial Diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals</td>
<td>29</td>
<td>2.6</td>
<td>0.9-6.25</td>
</tr>
<tr>
<td>Acute myelocytic leukemia at initial diagnosis</td>
<td>18</td>
<td>0.27</td>
<td>0.0-1.80</td>
</tr>
<tr>
<td>Acute myelocytic leukemia at initial diagnosis</td>
<td>1</td>
<td>23.1</td>
<td></td>
</tr>
</tbody>
</table>

The mean value for CFU-c in marrows of 29 normal individuals was 2.6, with a range of 0.9-6.26 colonies/10,000 marrow nucleated cells (Table 2). The mean value for colonies plated without HEKS was 0.6 colonies/10,000 marrow nucleated cells, with a range of 0.00 to 2.21.

**AML: Initial Values**

The pretreatment colony forming ability of 18 of 19 patients was uniformly poor (Table 2). For these patients the mean number of colonies/10,000 marrow nucleated cells plated with the colony-stimulating factor HEKS was 0.27 (range 0.00-1.80 colonies).

One patient, R.R., was distinctly different. Despite an untreated hypercellular bone marrow containing 90% leukemic myeloblasts, the initial in vitro colony production was 23.1 colonies/10,000 marrow nucleated cells (Table 2). The gross morphology of these colonies was normal and colony size was larger than normal, containing greater than 500 cells/colony at 10 days. Clinical data relating to presentation of disease or marrow morphology did not distinguish this patient from the 18 patients whose marrows produced poor colony formation. There was no cytogenetic abnormality noted in his direct marrow preparations.

**The Relationship of the Leukemic Myeloblast to CFU-c**

In 18 patients, excluding RR, 81 in vitro cultures were done. The relationship of the colony forming ability to the percentage of abnormal myeloblasts on each marrow smear is plotted in Fig. 1.

When the abnormal marrow blasts exceeded 40%, only two of 30 marrows produced colonies in the normal range. When the morphologically abnormal blasts occupied 20%, or less of the marrow, normal or greater than normal numbers of colonies occurred in 26 to 45 marrows. When the blast percentage was less than 5%, and hematologic remission was defined, most patients achieved normal or supranormal growth; however, even in this group, eight of the 22 marrows did not produce colonies within the normal range. Colony formation did not bear a linear relationship to the percentage of abnormal myeloblasts.

**Serial Studies of AML Patients**

To study in vitro growth during remission and relapse, serial studies were done on eight patients who achieved complete remission and could be followed by repeated marrow cultures. The durations of remissions ranged from 5 wk to 8+ mo. The remissions were less than 8 wk in three patients and longer than 5.5 mo in the remaining other five patients (Table 1). The pattern of serial colony forming ability was examined in these two groups.
The pattern of colony growth for the favorable group of patients with clinical remissions lasting greater than 5 mo can be illustrated by data from patient DW in Fig. 2.

Both stimulated and unstimulated values were low at initial diagnosis. At the time of remission the number of colonies of stimulated cultures was greatly increased and often exceeded the normal range. Subsequently, the CFU-c remained in or exceeded the normal range. These patients have remained in stable clinical and hematologic remission with normal colony growth for 5.5 to 9+ mo.

The single patient, R.R, Fig. 3, whose initial marrow culture supported exuberant growth at the time of diagnosis, was included in this category. At the time of initial marrow and prior to the second chemotherapy course, both the stimulated and unstimulated colony growth of this patient's marrow was extremely high. Morphologic remission was achieved after his third chemotherapy course, and following this remission his colony formation declined to the normal range. For the next 5 mo the marrow blasts ranged from 5% to 8%. Colony formation decreased and remained poor for the 3 mo prior to hematologic relapse. At the time of relapse, despite marrow morphology indistinguishable from his initial marrow, the exuberant colony growth of his early disease was not observed.
In the favorable group of five patients, three have eventually relapsed. Decreased colony growth was noted prior to relapse in two patients and coincidental with relapse in the third patient. The two remaining patients in sustained remission continue to have normal numbers of colony forming cells.

The pattern for the three patients with short remissions is illustrated by patient FD in Fig. 4. This patient, despite attaining a clinical and hematologic remission, never produced colony numbers that reached the normal range. The patient relapsed within 6 wk of achieving clinical and hematologic remission. The other two patients had similar patterns of poor colony formation during their brief remission periods. The serial CFU-c either entered the normal range only transiently or not at all.
Table 3. Colonies/10⁴ Cells Related to Remission Duration

<table>
<thead>
<tr>
<th>Remission Duration</th>
<th>No. Patients</th>
<th>Average Colonies/10⁴ Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 2 mo</td>
<td>3</td>
<td>0.13, 0.38, 2.28</td>
</tr>
<tr>
<td>≥ 6 mo</td>
<td>5</td>
<td>1.18, 3.17, 4.37, 5.8, 6.3</td>
</tr>
<tr>
<td>Normals</td>
<td>29</td>
<td>2.6 (9–6.25 range)</td>
</tr>
</tbody>
</table>

The mean number of colonies/10,000 marrow nucleated cells produced by 29 normals is 2.6, with a range of 0.90 to 6.25. By averaging the number of colony forming units produced during hematologic remission for each patient, a number is produced that correlates with remission duration (Table 3). Three patients who relapsed within 2 mo produced average values of colonies/10,000 marrow nucleated cells below the normal mean (Table 3). The average remission values of colonies/10,000 marrow nucleated cells of four of five patients whose clinical and hematologic remissions lasted more than 6 mo exceeded the normal mean of 2.6 (Table 3). The values that differentiate early and late relapse are significant for a p value of <0.05 by the Wilcoxon rank test.

DISCUSSION

Granulocytopenia is a common presenting manifestation of AML. Replacement of the normal marrow elements by leukemic blasts occurs as a hallmark of this disease. Return of the normal myeloid elements seldom occurs unless the proportion of leukemic myeloblasts is reduced to less than 10⁶. It is difficult to explain both the phenomena of leukemic cell overgrowth and the return of normal elements when the leukemic blasts numbers are successfully reduced in number. A more rapid accumulation of the abnormal cell line does not appear to be a cause because kinetic data indicates that the leukemic blasts have cell cycle times no shorter than the normal myeloid elements. The in vitro granulocyte colony assay provides a measure of the proliferative activity of the normal granulocyte precursor. It also allows the assessment of possible cell to cell interaction between the normal committed granulocyte precursors and the leukemic blasts.

The data in the current study provides some clues as to how the normal marrow elements are affected by the leukemic cell lines. In the current study, 18 of 19 patients failed to produce normal numbers of in vitro granulocyte colonies at the time of initial diagnosis. These observations are consistent with reports from other investigators. When clinical remission is attained and leukemic blasts are reduced to 5⁶, colony forming ability may return to normal. This data suggests that the blasts interfere with the colony forming activity of granulocytic progenitor cells. The leukemic myeloblasts decrease the colony forming ability of the granulocyte when the blast numbers exceed 20⁶ (Fig. 1). The leukemic myeloblasts inhibit colony formation in a nonlinear fashion similar to results reported from our laboratory for acute lymphocytic leukemia. The mechanism for this inhibition may be approached by combining cell separation techniques, cellular mixing experiments, or the addition of humoral inhibitor factors prepared from leukemic cells or sera.

Sequential studies demonstrated that, in remission, normal in vitro colony
formation ensued in five of the eight patients studied. The observation of normal numbers of granulocyte colonies appears to be a parameter differentiating those patients whose remissions are more stable from those patients relapsing within a 2-mo period.

While the marrow of most AML patients with florid disease does not form in vitro colonies, there is evidence that in some patients leukemic cells may proliferate and differentiate in the in vitro system.\textsuperscript{9,10} The description of small abortive colonies in patients with leukemia may represent a limited ability of some cells to proliferate.\textsuperscript{1,2} We have previously presented evidence that cytogenetically abnormal cells can differentiate to form colonies in the methylcellulose system.\textsuperscript{5} Patient RR appears to be an individual whose marrow at initial diagnosis was able to produce large numbers of normal-appearing colonies. Whether these colonies were produced from abnormal cells was not identified by cytogenetic study as his cell lines did not contain a marker.

Metcalf has suggested that leukemia may be a disorder of hemopoietic regulation, and that some leukemic cell populations are susceptible to differentiation and regulation under certain in vitro conditions.\textsuperscript{10} Sachs has demonstrated that murine leukemic cells have potential to differentiate in vitro with appropriate CSF.\textsuperscript{11} Thus, it may be possible to use an in vitro system to search for such susceptible leukemic cell populations and the development of growth regulators for clinical use. For those patients with tumors capable of differentiation, in vitro replacement of the growth regulators may cause the abnormal cells to differentiate along normal cell lines in vivo.

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REFERENCES

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