The Heritable Nature of Clonal Characteristics in Acute Myeloblastic Leukemia

By E. A. McCulloch, R. N. Buick, J. E. Curtis, H. A. Messner, and J. S. Senn

Great patient-to-patient variation has been observed when culture methods were used to study hemopoietic progenitor cells in patients with acute myeloblastic leukemia (AML). Using the double agar method of Robinson et al., abnormal growth patterns have been identified and prognostic significance applied to them. These abnormal growth patterns have been observed to become normal in remission and to recur in relapse; but quantitative data have not been reported for individual patients studied repeatedly. Marked variation in numbers of granulopoietic or erythropoietic colonies was seen when AML marrow was cultured in methylcellulose in the presence of leukocyte conditioned medium (LCM); with this technique, colony number was found to vary with time in a manner unrelated either to values at presentation or to clinical status.

The origin of patient-to-patient variation is relevant to the interpretation of culture results obtained using the methylcellulose technique. Noting both the distributions of the values of granulopoietic and erythropoietic progenitors among AML patients and the lack of consistency of the measurement in individuals with time, we have questioned the relevance of the measurements to cellular processes specific to leukemia; we have suggested that the data might be interpreted in light of findings suggesting that hemopoiesis may be predominantly clonal in leukemia. From this point of view, the patient-to-patient variation might be likened to the clone-to-clone heterogeneity observed when the cellular composition of individual spleen colonies is measured. In the latter instance, stochastic events occurring during clonal expansion have been proposed as the generators of heterogeneity. If a similar mechanism is responsible for patient-to-patient variation in the concentrations of myelopoietic progenitors, these values would reflect clonality rather than important leukemia-related abnormalities of differentiation. This view was supported by the clinical observation that measurements of progenitor cell concentrations obtained using the methylcellulose method did not contribute significantly to the variance in outcome of attempted remission induction.

As an alternative to measuring only progenitor cell concentrations, we have taken the approach of obtaining quantitative data on the properties of the blast progenitors in AML. Using a colony assay for this population, we now measure the following: capacity for self-renewal as determined by replating in culture, and sensitivity to adriamycin or cytosine arabinoside determined by exposure to drug in vitro. Marked patient-to-patient variation was again observed. We investigated the nature of this heterogeneity by making serial measurements. We have now studied seven AML patients on more than one occasion. Our findings indicate that self-renewal capacity and drug sensitivity are stable blast progenitor properties in each patient. As such, these properties may reflect the intrinsic nature of individual AML clones.

MATERIALS AND METHODS

Patients and Treatment

Peripheral blood was obtained in heparinized syringes from seven patients with AML repeatedly during the course of their disease. Table I contains hematologic data for the patients at each presentation together with a statement of clinical status and treatment. A number of chemotherapeutic protocols were used: CAV (cyclophosphamide, arabinosyl cytosine and vincristine), AAC (adriamycin, arabinosyl cytosine) and HU-Ara-C (hydroxyurea and arabinosyl cytosine) have been published. POMP consists of prednisone, oncovin, methotrexate, and 6-mercaptopurine. Other regimens include high-dose prednisone, methotrexate, vincristine, and 6-mercaptopurine or 6-thioquanine (6-TG) or 5-azacytidine (5-Aza). The protocol for obtaining blood for experimental studies was

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Blast Progenitor Assay

Blast progenitors were assayed using a modification\(^6\) of the method described by Buick et al.\(^4\) Mononuclear cells, obtained from peripheral blood by centrifugation through Ficoll-Hypaque at a density of 1.077 g/ml, were depleted of T lymphocytes by a second centrifugation through Ficoll-Hypaque after the rosette formation with sheep red blood cells (SRBC). The remaining leukocytes, containing blast populations, were plated in 0.8% methylcellulose containing 20% fetal calf serum (FCS), a-MEM (growth medium), and colonies enumerated using an inverted microscope. The results of the assay were confirmed by Wright-Giemsa stains and by routine morphological methods. Cultures containing blast cells were incubated for 10 days in a moist atmosphere at 37°C. This permitted repeated measurements of the colony-forming potential of the blast population. Simple negative exponentials are characterized by a dose-response curve obtained could be approximated by a simple negative exponential. This dose–response curve is characterized by the dose required to reduce colony formation to 10% of control levels (Adria D\(_{10}\)). For 52 AML patients, the D\(_{10}\) Adria value has varied from very sensitive (<0.1 μg/ml) to resistant (>5 μg/ml).

Measurement of Adriamycin Sensitivity

The sensitivity of blast progenitors to adriamycin was measured as described by Buick et al.\(^6\) Blast cells are exposed for 10 min to increasing concentration of the drug, washed twice, and plated for colony formation. A dose-dependent decrease in colony formation is observed with increasing concentration of adriamycin. The dose–response curve is characterized by the dose required to reduce colony formation to 10% of control levels (Adria D\(_{10}\)). For 42 AML patients, the D\(_{10}\) Adria value has varied from very sensitive (<0.1 μg/ml) to resistant (>5 μg/ml).

Sensitivity to Cytosine Arabinoside

Sensitivity of blast progenitors to cytosine arabinoside was measured using the method described by Niho et al.\(^7\) Blast progenitors were cultured for colony formation in the presence of increasing concentrations of cytosine arabinoside. A dose-dependent decrease in colony formation was observed; in most instances, the dose–response curve obtained could be approximated by a simple negative exponential (Fig. 1). In a small number of instances (approximately 10%), a tail in the dose–response curve was observed indicating a resistant population. Simple negative exponentials are characterized by the dose required to reduce survival to 10% (Ara D\(_{10}\)). For 42 AML patients, the Ara sensitivity has varied from very sensitive (Ara-C D\(_{10}\) < 10\(^{-3}\)M) to resistant (Ara-C D\(_{10}\) > 10\(^{-3}\)M).

Computation of D\(_{10}\) Values

D\(_{10}\) values for Adria and Ara-C were determined from the slopes of negative exponential curves determined using either a Hewlett Packard hand-held calculator or the University of Toronto Computer Centre. The authors are grateful to Dr. Larry Chang for writing programs for both computers.

RESULTS

Seven patients were studied at intervals of from 1 to 16 mo. In 2 patients studies were made before and after complete remission; in the remaining 5 patients extensive cytoreduction, but no remission, was achieved.
Repeated measurements on the same patient showed much less variation than that observed between patients. Figure 1 illustrates the point; the figure contains the data for repeated measurements of Ara-C sensitivity on 2 patients. Although Ara Dₐ₀ of blasts from the two patients varied almost tenfold, similar dose–response curves were obtained from each at every examination.

All the available culture data is given in Table 2. For PE2, values varied from 0 to >700 among 44 AML patients; in contrast, similar PE2 values were seen when the same patient was assessed repeatedly.

For Adria Dₐ₀ and Ara Dₐ₀, values among patients ranged from extremely sensitive to highly resistant. For these attributes, as for PE2, little variation was observed in repeated measurements on the same patient.

In only two patients were changes observed that might indicate progression. In patient 2, both PE2 and Adria Dₐ₀ were increased in a final sample taken just before death, while both properties had remained unchanged over the previous year. Patient 7 was treated with AAC and entered remission. The Ara-C survival curves at presentation and relapse after 12 mo are shown in Fig. 2; the data indicate that blast progenitors in this patient may have become less sensitive to Ara-C in the interval, although the values of PE2 and Adria Dₐ₀ were similar to those seen at presentation.

**DISCUSSION**

The major conclusion of this study is that the properties of blast cell renewal and blast cell sensitivity to adriamycin or cytosine arabinoside are stable properties of the leukemic clone in each AML patient. Complete remission was seen in 2 of the 7 patients and marked cytoreduction occurred in the others; therefore, repeated quantitative determinations of the three properties provides evidence that populations of blast progenitors emerging during clonal reexpansion expressed phenotypic characteristics similar to those present before treatment. It follows that the variation in these properties observed among the leukemic patients may reflect heritable clonal characteristics; as such, evaluation of properties differs from measuring primary plating efficiencies of progenitors, since the latter vary with time in response to physiologic factors or random events occurring during clonal expansion.¹⁰

Such stable or slowly evolving characteristics of leukemic clones may reflect biologic features of leukemia in each patient that may affect outcome. Indeed, self-renewal, a property that may be related to clonal aggression, was found to be significantly negatively correlated with successful remission induc-
Table 2. Summary of Laboratory Data

<table>
<thead>
<tr>
<th>Patient*</th>
<th>Time† (mo)</th>
<th>Self Renewal</th>
<th>Drug Sensitivity</th>
<th>Drug Sensitivity</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>n PE2 (10⁶)</td>
<td>n PE2 (2 x 10⁶)</td>
<td>Dₐ₀ Adria (µg/ml)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>31 ± 13</td>
<td>55 ± 38</td>
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<td>+6</td>
<td>54 ± 34</td>
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<td>20</td>
<td>25</td>
<td>1 2.7</td>
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<td>0</td>
<td>53</td>
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<td></td>
<td>+12</td>
<td>54 ± 7.2</td>
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<td>1 2.6</td>
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<td>31</td>
<td>1</td>
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<td>28 ± 2.8</td>
<td>47 ± 22</td>
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<td>—</td>
<td>—</td>
<td>1 5</td>
</tr>
<tr>
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<td>93 ± 4.2</td>
<td>92 ± 4.9</td>
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<td>38</td>
<td>38</td>
<td>1 3.3</td>
</tr>
</tbody>
</table>

*Same patient designation used in Table 1.
†Time after the first (0 time) observation.
‡Secondary plating efficiency determined from 4 dishes plated with 10⁴ cells, expressed as colonies/10⁴.
§Secondary plating efficiency determined from 4 dishes plated with 2 x 10⁵ cells, expressed as colonies/10⁴.

As yet, no correlation has been found between any of the three attributes and other risk factors in AML (McCulloch, Curtis, and Messner, manuscript in preparation).

Evidence of developing drug resistance was observed in only two of the seven patients. This finding was unexpected, since the development of drug resistance is observed commonly in experimental systems. However, the time of observation in the present series was short; a longer time might be required to observe clonal evolution. In the absence of such observations, it would be inappropriate to conclude that the properties we have studied are invariant with time; rather, it seems more reasonable to consider that they may change slowly. It may be that the short natural history of AML, together with treatment policies that include maintenance therapy, reduce the probability of the emergence of resistant populations.

We believe that this is the first report of stable cellular characteristics in human leukemia as measured in short-term cell culture. The findings of association between heritable characteristics and the outcome of treatment provide evidence for a need to individualize treatment. Perhaps of equal importance, any model of AML must explain the generation of this marked and stable patient-to-patient variation.

ACKNOWLEDGMENT

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Fig. 2. Ara-C dose–response curves for AML blasts from patient 7, Table 1. (•) At presentation, (△) at relapse after a 12-mo remission.
REFERENCES

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