Marrow Culture Studies in Adult Acute Leukemia at Presentation and During Remission

By Paul C. Vincent, Ruth Sutherland, Margot Bradley, David Lind, and Frederick W. Gunz

Culture of bone marrow and/or blood cells in a semisolid agar system from 43 adults with acute nonlymphoblastic leukemia at first presentation showed two distinct growth patterns at 14 days. In 53% of patients cells failed to grow (type O), while in the remainder an abnormal growth pattern (type B) with small numbers of diffuse colonies and excessive numbers of cell clusters was seen. The response following chemotherapy was significantly better in the patients whose cells failed to grow. Serial culture studies, performed in 9 patients throughout remissions of 100–1112 days, which had been maintained by intermittent chemotherapy, showed wide fluctuations in proliferative activity. These ranged from no growth to marked proliferation with predominance of clusters and small numbers of diffuse colonies, indistinguishable from the type B pattern seen in 47% of patients at first presentation. The possibility is discussed that the periods of failure to grow, and/or those in which a type B pattern emerged, represented sporadic reactivation of leukemic cells.

Tissue culture of bone marrow cells in a soft gel medium has become a widely accepted technique for the study of human granulopoietic progenitors. In the presence of an appropriate stimulus (colony-stimulating activity, CSA) cells which are almost certainly committed stem cells proliferate and mature to give rise to colonies of granulocytes and/or monocytes. It is generally agreed that monocytes rather than granulocytes are the source of blood leukocyte CSA. Moore et al. have proposed a model in which a stem cell committed to either granulocyte or monocyte differentiation can be switched from one cell line to the other depending on the level of CSA present.

There have been many reports of the use of the agar culture technique to study the proliferation of blood and/or marrow cells from patients with acute nonlymphoblastic leukemia (ANLL) at the time of first presentation. Several distinct proliferative patterns have been described, which range from a complete failure to grow in culture, through distinctly abnormal growth patterns with predominance of cell clusters, to the occasional finding of apparently normal colony formation. Although there have been reports of normal cell maturation occurring from leukemic blasts in culture, most workers regard

*ANLL: acute myeloblastic leukemia (AML), acute myelomonocytic leukemia (AMML), acute promyelocytic leukemia (APL), acute erythroleukemia (AEL), or acute undifferentiated leukemia (AUL).

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the apparent differentiation seen in agar cultures as still being abnormal\textsuperscript{17,18} by contrast with the apparently normal maturation seen in the diffusion chamber system.\textsuperscript{19}

The relationship between normal and leukemic stem cells, both at the time the patient first presents and during complete remission, is poorly understood. At first presentation, there is good evidence to suggest that erythroid precursors are also derived from leukemic stem cells,\textsuperscript{20,21} and it seems likely that normal stem cells are suppressed or switched off.\textsuperscript{22} During complete remission, on the other hand, it is generally believed that normal hemopoiesis is due to the reactivation of normal stem cell activity, although leukemic cells persist and are responsible for ultimate relapse. In an attempt to define these relationships, we have used the agar colony technique to study patients with ANLL at first presentation and, where possible, serially throughout remission.

**MATERIALS AND METHODS**

**Patients**

Forty-three adult patients with ANLL were studied before treatment (Table 1). The patients ranged in age from 14 to 76 years and there were 23 females. At presentation, the proportion of leukemic cells ranged from 15\textsuperscript{o} to 100\textsuperscript{o} (median 81\textsuperscript{o}) in bone marrow and from 0 to 97\textsuperscript{o} (median 59\textsuperscript{o}) in blood. After initial studies, 34 patients were treated with hydroxyurea, cytosine arabinoside, and thioguanine,\textsuperscript{23} and 9 with hydroxyurea, cytosine arabinoside, and daunorubicin. Twenty-one patients went into complete remission, as judged by accepted criteria,\textsuperscript{23} and one into prolonged partial remission. As in our previous analyses,\textsuperscript{23,24} there was no correlation between age or initial blood leukocyte count and outcome. The absence of adverse effects due to a high white cell count in our experience was probably due to our use of hydroxyurea, which causes the white cell count to fall with a half-time of the order of 19 hr.\textsuperscript{24} Maintenance therapy, at least for the first 2 yr, consisted of 4-5 day courses of cytosine arabinoside and thioguanine every 4 wk.\textsuperscript{23}

In 28 patients, bone marrow and venous blood samples were cultured at the time of initial presentation, while in 10 only marrow, and in 5 only blood samples were cultured (Table 1). Bone marrow samples (obtained for assessment of progress before courses of intermittent maintenance therapy) were cultured throughout prolonged remission in 9 patients. Pretreatment marrow and/or blood cultures had been performed in 7 of these patients, while in the remaining 2 they were commenced during remission.

**Methods**

Marrow samples were cultured in a semisolid agar system at a concentration of \(2 \times 10^5\) nucleated cells per dish over feeder layers of \(1 \times 10^6\) normal blood leukocytes per dish, using the technique of Robinson and Pike.\textsuperscript{25} The numbers of leukemic cells in these cultures ranged from \(3 \times 10^5\) to \(2 \times 10^5\) (median \(1.6 \times 10^5\)), depending on the proportion in the marrow sam-

<table>
<thead>
<tr>
<th>Table 1. Culture Studies in ANLL at Presentation</th>
</tr>
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<tbody>
<tr>
<td>Diagnosis</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>AML</td>
</tr>
<tr>
<td>AMML</td>
</tr>
<tr>
<td>AUL</td>
</tr>
<tr>
<td>APL</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
Marrow samples from 64 hematologically normal patients without malignant disease undergoing general anesthesia for endoscopic or minor surgical procedures were obtained, with the patient’s voluntary informed consent, and cultures from these marrows served as normal controls.

RESULTS

Normal Values

Marrow samples from 64 hematologically normal controls, cultured over the same time period as these studies, gave rise to 36 ± 3 (mean ± SEM) colonies per 2 x 10^5 nucleated cells after 14 days. The mean ratio of clusters to colonies in these normal marrows, also at 14 days, was 2.0(±0.2):1. Normal blood leukocytes gave rise to 0–1 colony per 2 x 10^5 nucleated cells, with a similar cluster-to-colony ratio. In both marrow and blood cultures granulocytic pre-
Table 2. Culture Data (Marrow and/or Blood)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Growth Pattern</th>
<th>No Growth</th>
<th>Growth</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td></td>
<td>15</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>AMML</td>
<td></td>
<td>7</td>
<td>5</td>
<td>12</td>
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<tr>
<td>AUL</td>
<td></td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>APL</td>
<td></td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>23</td>
<td>20</td>
<td>43</td>
</tr>
</tbody>
</table>

cursors predominated up to 7 days, after which they were progressively replaced by mononuclear cells.

Leukemic Cultures at Time of Diagnosis

There was a close concordance between the pattern of growth (no growth, cluster formation only, or colonies and clusters) in blood and marrow cultures in the 28 cases where both were carried out; tested by goodness of fit, there was no significant difference between the two ($\chi^2 = 0.7214$, 2 df, $0.4 > p > 0.3$). (See Fig. 1.) For the 11 cases where colonies were found, there was a highly significant correlation between the number in the blood and the number in the marrow ($r = 0.9357$, $p < 0.001$, slope = 0.92).

Twenty-two of the 38 marrow samples and 18 of the 33 blood samples failed to show any growth (Fig. 1). There was no correlation between the type of growth pattern or the numbers of colonies and the proportion of leukemic cells in either marrow or blood. Although a growth pattern was more often seen in AUL and APL (Table 2), the number of patients was too small to determine whether this was significant. There was, however, a significant association between failure to grow in culture and response to treatment. Of the 23 patients who failed to show growth, 15 went into complete remission and 1 into partial remission, compared with 6 complete remissions in the 20 patients whose cells grew ($\chi^2 = 5.21$, $0.025 > p > 0.01$). (See Table 3.)

When growth did occur in culture, it showed a distinctly abnormal pattern, with excessive numbers of clusters, small numbers of diffuse colonies, and large numbers of single cells. This pattern, which we have designated type B, was associated with a mean cluster-to-colony ratio of 30(±16):1, and was distinctly different from that seen in cultures of normal marrow. Morphological examination of cells from these cultures showed the persistence of frankly leukemic cells or abnormal monocytoïd cells with convoluted immature nuclei. No normal mature granulocytes or monocytes were seen in any of these cultures.

Table 3. Acute Nonlymphoblastic Leukemia Culture Studies at Presentation

<table>
<thead>
<tr>
<th>Culture Pattern</th>
<th>Outcome</th>
<th>No Growth</th>
<th>Growth</th>
<th>Total</th>
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<tbody>
<tr>
<td>Complete remission</td>
<td>15</td>
<td>6</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Partial remission</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fail</td>
<td>7</td>
<td>14</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>20</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

$\chi^2$, C + P vs. Fail, NG vs. G = 5.2122, $0.025 > p > 0.01$. 
Table 4. Serial Studies During Remission in ANLL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Marrow Blood Marrow</th>
<th>Growth*</th>
<th>Remission Survival</th>
<th>Relapse Growth*</th>
<th>Clusters (\text{-}2\text{ Relapse})</th>
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</thead>
<tbody>
<tr>
<td>AM M</td>
<td>AML</td>
<td>O 0 46XY</td>
<td>C 100 B O</td>
<td></td>
<td></td>
<td></td>
<td>335 100</td>
</tr>
<tr>
<td>HM F</td>
<td>AML</td>
<td>NT NT 46XX</td>
<td>C 144 O O</td>
<td></td>
<td></td>
<td></td>
<td>309 76</td>
</tr>
<tr>
<td>JB F</td>
<td>AML</td>
<td>NT NT 46XX</td>
<td>C 262 NT NT</td>
<td></td>
<td></td>
<td></td>
<td>439 197</td>
</tr>
<tr>
<td>CN F</td>
<td>AML</td>
<td>O 0 46XX</td>
<td>C 279 LN B</td>
<td></td>
<td></td>
<td></td>
<td>489 28</td>
</tr>
<tr>
<td>DT M</td>
<td>AML</td>
<td>O 0 46XY</td>
<td>P 380 NT O</td>
<td></td>
<td></td>
<td></td>
<td>688+ 63</td>
</tr>
<tr>
<td>SG F</td>
<td>AML</td>
<td>NT O 46XX (+D – G)</td>
<td>C 504 NT NT</td>
<td></td>
<td></td>
<td></td>
<td>768 78</td>
</tr>
<tr>
<td>BC F</td>
<td>AML</td>
<td>NT B 46XX</td>
<td>C 981 — —</td>
<td></td>
<td></td>
<td></td>
<td>1022 —</td>
</tr>
<tr>
<td>EW F</td>
<td>AML</td>
<td>O 0 46XX</td>
<td>C 985 B NT</td>
<td></td>
<td></td>
<td></td>
<td>1209+ 112</td>
</tr>
<tr>
<td>TK M</td>
<td>AML</td>
<td>O 0 46X (+D – 2G)</td>
<td>C 1112 LN HN</td>
<td></td>
<td></td>
<td></td>
<td>1402 146</td>
</tr>
</tbody>
</table>

\*O, no growth; B, type B growth pattern (see text); NT, not tested; LN, low normal; HN, high normal.

\(\text{\textsuperscript{a}}\)Direct marrow preparation; 30 metaphases counted in each case. Patient SG had 27/30 46XX (+D – G), 1 47(+G), 1 45 and 1 46XX.

Patient TK had 20/30 45 (+D – 2G), 6 46XY, 3 45 (+D + 16 – C = 2G) and 1 45.

\(\text{IC, complete; P, partial.}\)

\(\text{\textsuperscript{b}}\)Time from peak of cluster growth to relapse. Patient CN in second remission; BC died while in complete remission.

Serial Studies

Nine patients were studied throughout remission (Table 4). Marrow cultures had been performed in five at first presentation, and all failed to grow, while in seven, cultures of blood leukocytes at first presentation had yielded a type B growth pattern in one and no growth in six. Cytogenetic studies of bone marrow cells at presentation yielded normal chromosomes in seven, a pseudodiploid pattern in one (SG) and a predominant hydodiploid clone with 45 chromosomes in one (TK) (Table 4). Banding techniques were not available at the

Fig. 2. Results of serial marrow cultures in a patient (EW) with AML during prolonged complete remission. Intermittent therapy with cytosine arabinoside and thioguanine\textsuperscript{23} (after marrow sampling) shown by arrows.
Fig. 3. Results of serial marrow cultures in a patient (TK) with AML during prolonged complete remission. Intermittent therapy with cytosine arabinoside and thioguanine (after marrow sampling) shown by arrows and continuous 6-mercaptopurine shown by shaded rectangles.

Fig. 4. Results of serial marrow cultures in a patient (BC) with AML during prolonged complete remission. The patient died of infection while in complete remission. Intermittent therapy with cytosine arabinoside and thioguanine (after marrow sampling) shown by arrows and continuous 6-mercaptopurine shown by shaded rectangles.
time these patients were initially studied. Serial marrow studies showed a normal chromosome complement during remission in all patients and no change from the initial chromosome pattern at relapse. Cytogenetic analyses were performed on marrow cultures from patients BC, TK, HM, and CN on one or two occasions each, and these yielded normal results, but serial studies were not carried out.

Seven patients were in complete remission throughout the study, for periods of 100-1112 days. One (patient CN) had been in complete remission for 279 days but was studied during a second brief complete remission from day 395 to day 423, and one (patient DT) was in a prolonged (380-day) partial remission. Maintenance therapy, at least for the first 2 yr, consisted of 4-5 day courses of cytosine arabinoside and thioguanine every month. Marrow samples for culture were obtained from aspirates performed before each course of chemotherapy. The results of these cultures showed marked variation, from no growth (when parallel normal marrow samples grew normally on feeder layers from the same source) to a striking proliferation, predominantly of clusters, but also of colonies, similar to the type B pattern described above (Figs. 2-4). The morphology of cells from these cultures was also indistinguishable from that found in cultures which grew from patients at first presentation.

The time course for each of the patients studied is shown in Fig. 5, where the sporadic occurrence of episodes of type B growth is indicated by arrows. Despite the similarities between this growth and that seen in some patients at first presentation, these patients remained in remission and each episode was followed by a return to normal or even subnormal growth in culture. However, in each case where relapse occurred it was possible to identify a cluster “peak” 28-197 days earlier (Table 4).

Of the five marrows cultured from these patients at the time of relapse, two showed a type B pattern, two a low colony count with few clusters (“low normal”), and one failed to grow. Of the six bloods cultured, three failed to grow, one showed ten colonies and six clusters per 2 x 10^6 cells, (“high normal”), and one showed a type B pattern (Table 4).

DISCUSSION

Our data, like those of Moore et al., indicate that the results of in vitro marrow culture from ANLL patients at presentation are of prognostic value in
assessing likely response to treatment. The differences in detail between our results and those of Moore et al.\textsuperscript{15} are due to differences in the period of culture. They have found the best response to treatment in patients who grow small clusters after 7 days, and in our more recent experience cultures which show this pattern at 7 days usually show no growth after 14 days. It is not immediately clear why patients whose marrow fails to grow to any degree should do better with treatment.

There is good evidence to suggest that colonies which do grow in culture from leukemic marrow or blood are of leukemic origin,\textsuperscript{14,15,17,18,25,26} and will only grow in the presence of a stimulus provided either by normal leukocytes (or conditioned media prepared from them)\textsuperscript{11,12,14} or by adherent marrow cells,\textsuperscript{27,29} which is probably the same as that required for normal granulopoiesis.\textsuperscript{27} Leukemic cells capable of responding to these stimuli might also be stimulated by granulopoietin in vivo, and might be more difficult to suppress by chemotherapy as a result. On the other hand, leukemic cells\textsuperscript{30} and leukemic neutrophils\textsuperscript{31} inhibit normal colony growth in vitro, and interaction between inhibition and stimulation in vivo might also be important in determining response to treatment.

A recent report by Tebbi et al.\textsuperscript{32} described no correlation between blood and marrow culture results in 17 patients with AML and 1 with an acute transformation of chronic granulocytic leukemia either in relapse or in remission. There was no mention of cultures growing clusters only, although aggregates of more than 20 cells were scored as colonies.\textsuperscript{33} In our series, however, we did find a significant concordance between the growth pattern (i.e., no growth, clusters, or colonies) from blood and marrow in 28 patients with AML at first presentation, and a significant correlation between the numbers of colonies in blood and marrow in the 11 patients where colonies were found. The differences probably relate to technique and classification of cultures, but remain to be resolved. Likewise, we did not see the continuous spectrum of increasing colony counts reported by Curtis et al.\textsuperscript{34} in unstimulated leukemic marrows, but our cultures were all stimulated by normal leukocyte feeder layers. When the data of Curtis et al. for stimulated leukemic marrow were reviewed, the continuity of any spectrum was much less obvious.

The sporadic occurrence of abnormal growth patterns during complete remissions was a striking finding. Galbraith\textsuperscript{35} also observed this in three patients, although other studies in which only colonies but not clusters were enumerated failed to detect it.\textsuperscript{36,37} On culture pattern and morphology, the growth was like that seen in nearly half of the cases of acute leukemia at first presentation, and the possibility that it arose from the proliferation of leukemic cells during remission has to be considered. Equally, of course, it could be argued that the sporadic failure of marrow to grow during remission was an expression of residual leukemic cell activity, particularly since this had been the pattern at initial presentation in all but one of the long-remission patients. Cytogenetic studies would have helped decide whether or not the sporadic type B growth patterns were leukemic or not, but unfortunately these were not performed. As it turned out, only two of the long-term survivors had recognizable chromosome abnormalities at presentation, and cytogenetic studies would not have
helped resolve the question in the remainder. Without these, one could not prove that these episodes of type B growth were due to the persistence of leukemic cells. However, the type of proliferation and the morphology of the cells were both distinctly abnormal, and were quite unlike the pattern we have seen in regenerating normal marrow, where there was an increased number of compact colonies with a normal cluster-to-colony ratio and normal cell morphology.

The virtual inevitability of relapse in ANLL makes it almost certain that leukemic cells persist throughout remission, probably in a G0 state. The relationship between these resting leukemic cells and the stem cells responsible for apparently normal hemopoiesis during remission is not clear, but it seems possible that chemotherapy and the host’s own defenses serve to keep the leukemic cells suppressed. Under these circumstances, it would not be surprising to find sporadic leukemic cell reactivation from time to time, and it is conceivable that either the episodes of type B growth, or the periods of failure to grow, or both, reflect this. In long survivors, several of these episodes could occur without relapse if the emergent clone is susceptible to chemotherapy and/or the patient’s own defense system.

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Marrow culture studies in adult acute leukemia at presentation and during remission

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