ABSTRACT

Bone marrow cells obtained from 166 patients with acute nonlymphocytic leukemia were cloned in vitro. The number and size of clones produced differed among patients and was unrelated to French-American-British type of leukemia, to patient age, to whether the patient was studied at the time of initial diagnosis or at relapse, or to the cytogenetic (normal or abnormal metaphases) or cell cycle characteristics of the leukemic bone marrow cells. The ability of leukemic cells to clone in vitro was associated with poor response to therapy in vivo, with the remission rate or at relapse, or to the cytogenetic (normal or abnormal metaphases) or cell cycle characteristics of the leukemic bone marrow cells. Measurement of the clonogenic potential in vitro of leukemic marrow cells together with their cytogenetic type may help to distinguish between patients who should and should not receive cytosine arabinoside/anthracycline antibiotic remission induction therapy and patients who do and do not require intensive remission consolidation chemotherapy.

MATERIALS AND METHODS

Acquisition of Cells. Bone marrow cells obtained from 166 patients with ANLL were studied. The patients’ diagnoses met the criteria established by the FAB working party (1). Marrow was aspirated into a syringe containing 2 ml of 10% sodium citrate, and interface cells were collected after centrifugation of the marrow cell suspension through Ficoll-Paque (specific gravity, 1.077) (Pharmacia Fine Chemicals, Piscataway, NJ) at 400 x g for 30 min at room temperature. The cells were washed with RPMI 1640 tissue culture medium (Grand Island Biological Co., Grand Island, NY), made 10% with fetal calf serum (Flow Laboratories, Inc., McLean, VA), and used for study.

Cloning Methods. Granulocyte/macrophage colony forming units were assayed as reported previously (16). In brief, 1 x 10^6 cells were mixed with 0.85 ml of growth medium consisting of 0.3% agar (Difco Laboratories, Inc., Detroit, MI), 20% fetal calf serum in Eagle’s tissue culture medium (Grand Island Biological Co.) and then plated over a 2.5-ml underlayer prepared in a 35- x 10-mm plastic dish (Lux Scientific Corp., Newbury Park, CA). The underlayer consisted of 0.5% agar, 20% fetal calf serum, Eagle’s tissue culture medium, and 10% grant cell tumor-conditioned medium as a source of colony-stimulating activity (4). The cultures were plated in triplicate and incubated for 7 days at 37° in a humidified atmosphere consisting of 5% CO₂ and 95% room air. The cultures were counted with an estimate made by eye of the number of cells/clone, and the clones were grouped into those consisting of 4 to 10 cells, 11 to 20 cells, 21 to 40 cells, or greater than 40 cells (11). The morphology of the cells within the clones were evaluated for the first 15 patients studied and, as we have reported previously (9), the cells within the clones were either similar in appearance to the cells which were plated or appeared to have undergone abirritive myeloid maturation. These culture conditions do not support the growth of either normal or leukemic lymphocytic cells (9).

INTRODUCTION

Bone marrow cells obtained from some patients with ANLL will produce clonal growth in vitro under conditions which are suitable for the growth of normal granulocytic progenitor cells (11). The growth pattern of marrow cells obtained from patients with ANLL has been reported to be of prognostic significance with regard to the outcome of remission induction therapy (2, 5-8, 11, 22). In the study reported here, marrow specimens were obtained from 166 patients with ANLL and were cultured in vitro in agar. The relationships between the growth pattern in vitro and the clinical characteristics of the patients, as well as between growth in vitro and the morphological, cell cycle, and cytogenetic characteristics of the leukemic cells, were sought. Additionally, we studied the relationship between both the outcome of remission induction therapy and the duration of remission and the growth characteristics of the leukemic cells.

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The abbreviations used are: ANLL, acute nonlymphocytic leukemia; FAB, French-American-British working party classification of acute nonlymphocytic leukemia; dThd, thymidine; ara-C, 1-β-D-arabinofuranosycytosine; NH, bone marrow specimens in which normal metaphases were detected; NA, bone marrow specimens containing both normal and abnormal metaphases; AA, bone marrow specimens in which only abnormal metaphases were detected; CR, complete remission; RD, remission induction failure due to persistent leukemia.

H. D. Preisler, N. Azarnia, and M. Marinello, unpublished observations.
present study, patients were considered to have NN leukemia if only normal metaphases were detected. A patient was considered to have AA leukemia if only abnormal metaphases were noted, and a patient was considered to have NA disease if there were 2 or more identical abnormal metaphases which contained the same structural abnormalities or the same additional chromosomes and at least a single normal metaphase. In the case of hypodiploidy, 3 or more metaphases missing the same chromosome(s) constituted a clone. This simple cytogenetic classification, while not distinguishing between the specific clonal abnormalities which may be present, provides clinically meaningful information with respect to the outcome of remission induction therapy, with NN patients having the highest and AA patients having the lowest CR rates (7, 15, 18, 20).

Clinical Aspects. The patients were treated according to RPMI Protocol 970701 (Chart 1A) or Protocol 998028 (Chart 1B) or with high-dose ara-C (3 g/sq m every 12 hr x 12 doses). The remission induction regimen used in Protocols 97071 and 998028 were essentially identical, but the duration of consolidation/maintenance therapy differed, as indicated in Chart 1. The patients who were induced into remission with high-dose ara-C did not receive maintenance therapy.

A CR was defined according to the criteria proposed by Cancer and Acute Leukemia Group B (12). In brief, the bone marrow had to contain <5% myeloblasts or leukemic cells with evidence of normal hematopoiesis and normal peripheral blood cell counts. Patients whose leukemia entered CR were considered to have drug-sensitive disease. Patients who did not enter CR were classified according to failure type so that a determination could be made as to whether or not clinically documented drug-resistant disease was present (15, 19). Bone marrow aspirates and biopsies were carried out 7 days after the conclusion of a course of remission induction therapy. Patients were considered to have drug-resistant disease if the marrow aspirate demonstrated persistent leukemic cells and the biopsy cellularity was >5% or if the 7-day-posttherapy marrow was devoid of leukemic cells but leukemic cells subsequently repopulated the bone marrow. Patients who expired before the 7-day-posttherapy marrow could be examined or who expired with a hypocellular bone marrow were considered to be inevaluable with respect to the presence of drug-sensitive or drug-resistant disease and were labeled as “other failures.”

Analysis of Data. The studies of the relationship between the total number and size of the largest colony/cluster and the outcome of remission induction therapy were carried out both for previously untreated patients and for relapsed patients. In both cases, the outcome was divided into CR and remission failure. The failures were further divided into drug-resistant disease failures or “other” failures. χ² values were computed for each case, and the corresponding p-values were determined.

Both Kruskal-Wallis and Mann-Whitney tests were used to compare the cloning efficiency for different cytogenetic types. The relationship of both cloning efficiency and the average size of clones produced with FAB type was investigated by Kruskal-Wallis tests. In general, we have used distribution-free statistics in univariate analyses (21), except for the correlation coefficients, which are Pearson’s r coefficients.

The proportional hazards model of Cox (3) was used to provide a regression model for relapse rate based on maximum colony/cluster size. The actual relationship of relapse time and maximum colony/cluster size was investigated by Kaplan-Meier curves (Chart 5), and median duration of remission was calculated.

Finally, multivariate logistic regression was used to find the logistic function of independent variables that best distinguish between various outcomes.

RESULTS

General Characteristics of Leukemic Cell Growth in Vitro

The growth characteristics of leukemic marrow cells obtained from 90 previously untreated patients and from 76 patients with relapsed leukemia were assessed. The total number of clones produced by the different specimens ranged from 0 to 4585/10⁴ cells plated with a median of 60 clones. Marrow specimens from 22% of the patients failed to produce any growth in vitro. For 12% of the specimens, growth was restricted to clones consisting of <10 cells; 26% of the specimens produced at least one clone consisting of 10 to 20 cells, and 17 and 22% of the specimens, respectively, produced at least one clone of 20 to 40 cells or >40 cells. Smaller clones were more likely to be associated with specimens which produced <20 clones than with specimens which produced >20 clones (p < 0.001).

The growth pattern of marrow cells obtained from 90 previously untreated patients was compared with that of marrow obtained from 76 patients who had relapsed leukemia. Marrow from previously untreated patients produced 392 ±61 (S.E.) clones, with a median value of 61. The comparable figures for patients with relapsed leukemia was 327 ± 66, with a median value of 59. The marrow of 26% (23 of 90) of previously untreated patients failed to produce even a single clone in vitro, while the comparable figure for the relapsed patients was 18% (14 of 76). The differences in the proportion of nongrowers for these 2 groups is not statistically significant. The size distributions of the clones were similar for both groups, with the average clone size of 8.6 ± 0.6 and 8.9 ± 0.6 cells for specimens obtained from previously untreated patients and from patients with relapsed leukemia, respectively.

[3H]dThd suicide indices were available for 83 specimens. Forty-eight of the specimens were obtained from newly diagnosed patients, and 35 were from relapsed patients. Chart 2A

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index of the marrow specimen and the size distribution of the clones produced by the specimen. As with the suicide indices, the labeling indices of relapsed patients tended to be less than that for previously untreated patients (median values, 8 and 9.2%, respectively), but these differences were not statistically significant ($p < 0.5$).

FAB classification was available for 146 of the cloned specimens. The in vitro growth patterns of the different FAB types were compared with respect to: growth versus no growth; total clonal growth; and the maximum size of the clones produced. The proportion of nongrowers ranged from 20 to 40% and was not significantly different among the different FAB types. Chart 3 and the accompanying data in the chart legend provides information regarding total growth, median values for growth, and average size of clones. These data demonstrate that there was no demonstrable relationship between FAB type and the in vitro cloning ability of the cells. Comparison of the growth pattern of the different FAB types at initial diagnosis or at the time of

![Chart 2](chart2.png)

**Chart 2.** Relationship between the percentage of cells in S phase and the cloning efficiency in vitro of leukemic bone marrow cells. A, $[^3H]dTdR$ suicide index versus cloning efficiency (no. of clones per $10^5$ cells plated); B, $[^3H]dTdR$ labeling index versus cloning efficiency. Numbers on graph indicate the number of specimens at that point.

The chart illustrates that there was no relationship between the suicide index and the number of clones produced ($r = 0.025$). Clonal growth was produced by 13 specimens for which the suicide index was 0, while low numbers of clones were produced by some specimens the suicide indices of which were as high as 98%. There was no demonstrable relationship between the suicide index and average size of the clones produced *in vitro* or with the size distribution of the clones. The $[^3H]dTdR$ suicide indices for specimens obtained from 48 previously untreated patients and 35 patients with relapsed disease were compared, with the latter values being somewhat less than the former (median values, 48 and 35%, respectively), but this difference was not of statistical significance. $[^3H]dTdR$ labeling indices were available for 95 specimens. Chart 2B illustrates the relationship between labeling index and total growth ($r = -0.09$). The mean labeling indices for nongrowers and growers were 7.7 ± 1.7 and 9.9 ± 9, respectively, and the medians were 7.3 and 9, respectively. There was no discernible relationship between the labeling

![Chart 3](chart3.png)

**Chart 3.** Cloning characteristics in vitro of acute nonlymphocytic leukemic bone marrow cells according to FAB type. A, cloning efficiency; B, average size of clones produced. Numbers on graph indicate the number of specimens at that point.
relapse demonstrated no significant differences. [3H]dThd labeling indices and suicide indices were compared for the different FAB types, and no differences were found (data not shown). Additionally, there was no relationship between the percentage of myeloblasts and/or promyelocytes in the marrow specimen and the number of clones produced in vitro (r = 0.1234).

Cytogenetic studies were carried out on 63 of the marrow specimens prior to plating in vitro. Thirty-four patients were classified as NN, 14 as NA, and 15 as AA. Chart 4 and the data accompanying the chart demonstrate that the cloning efficiencies of the marrow cells were identical for the different cytogenetic types. The size distributions of the clones produced in vitro by the cytogenetic subtypes were analyzed, and it was found that AA cells were less likely to produce clonal growth of 40 cells. Eleven of the 34 NN specimens and 5 of the 14 NA specimens produced at least one clone consisting of >40 cells, while this was true for only one of the 15 AA specimens (p = 0.073 for NN versus AA; p = 0.083 for NA versus AA). With respect to treatment status, 29 of the NN, 12 of the NA, and 10 of the AA patients were studied at the time of initial diagnosis. The lack of relationship between cytogenetic type and in vitro growth pattern was true for patients studied at the time of initial diagnosis or at the time of leukemic relapse.

Relationship of Marrow Growth In Vitro and Patient Characteristics. A relationship was sought between in vitro growth characteristics and patient age. The following parameters were analyzed: growth versus no growth; total number of clones produced; and size distribution of clones. The median age for patients whose marrow specimens failed to clone in vitro was 51 years, while the corresponding value for patients whose specimens cloned in vitro was 57 years. The correlation coefficient between age and total growth in vitro was 0.034, while the correlation between age and average size of cluster/colonies was −0.040. The same analyses were carried out regarding the sex of the patients and, once again, no relationships were discernible.

Relationship between Marrow Growth Characteristics In Vitro and Response to Chemotherapy. Two parameters of response to therapy were considered: (a) outcome of the course of chemotherapy which was administered immediately after the in vitro study; and (b) remission duration.

The relationship between the growth characteristics in vitro of leukemic marrow cells and the outcome of therapy was investigated from several perspectives. With respect to in vitro growth pattern, the total number of cluster/colonies formed and the maximum size of the cluster/colonies was compared to treatment outcome. Treatment outcome itself was considered from 2 aspects: the outcome of remission induction therapy and remission duration.

The data provided in Table 1 demonstrate the relationship between in vitro growth characteristics and the outcome of remission induction therapy. While patients whose leukemic cells grew well in vitro (>100 cluster/colonies or a maximum size of cluster/colonies >20 cells) tended to have lower remission rates (CR rates of 44% for patients in either category) than did patients whose leukemic cells grew less well in vitro (the CR rate for patients whose leukemic cells produced <100 colonies/clusters was 69%, and the CR rate for patients whose leukemic cells didn’t produce a single cluster consisting of >20 cells was 67%), these differences were not statistically significant. Similar observations were made for relapsed patients (Table 2). Of interest was the fact that, when failure type was considered, failure due to drug resistance or induction death ("other failure") was equally represented, regardless of the in vitro growth pattern of the leukemic marrow cells.

**Table 1**

<table>
<thead>
<tr>
<th>Growth pattern</th>
<th>CR failures</th>
<th>&quot;Other&quot; failures</th>
<th>p</th>
<th>(CR vs. no CR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No growth</td>
<td>15 (68)</td>
<td>3 4</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>&gt;0 and ≤100 cluster/colonies</td>
<td>14 (70)</td>
<td>2 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;100 cluster/colonies</td>
<td>15 (44)</td>
<td>11 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. No growth</td>
<td>15 (66)</td>
<td>3 4</td>
<td>0.186</td>
<td></td>
</tr>
<tr>
<td>&gt;0 and ≤20 cells</td>
<td>16 (62)</td>
<td>6 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20 cells</td>
<td>13 (45)</td>
<td>7 9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Numbers in parentheses, percentage of CR.
b 2 x 2 square analysis of the relationship of growth characteristics in vitro and outcome of therapy.
c Total number of cluster/colonies.
d Maximum size of cluster/colony.

**Table 2**

<table>
<thead>
<tr>
<th>Growth pattern</th>
<th>CR failures</th>
<th>&quot;Other&quot; failures</th>
<th>p</th>
<th>(CR vs. no CR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No growth</td>
<td>4 (31)</td>
<td>4 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;0 and ≤100 cluster/colonies</td>
<td>8 (25)</td>
<td>13 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;100 cluster/colonies</td>
<td>5 (31)</td>
<td>5 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. No growth</td>
<td>4 (31)</td>
<td>4 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;0 and ≤20 cells</td>
<td>10 (38)</td>
<td>7 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20 cells</td>
<td>3 (17)</td>
<td>11 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Numbers in parentheses, percentage of CR.
b Total number of cluster/colonies.
c Maximum size of cluster/colonies.
to maximum colony/cluster size, in that the larger the cluster/colony, the shorter the duration of remission ($p = 0.0048$). Chart 5 presents these data. The median durations of remission for patients whose cells failed to clone in vitro, for patients whose marrow cells produced clusters the maximal size of which was <20 cells, and for patients whose marrow cells produced at least 1 cluster/colony consisting of ≥20 cells were 93, 47, and 27 weeks, respectively. In contrast, remission duration was unrelated to the total number of cluster/colonies which were formed ($p = 0.8370$), and inclusion of both the maximum cluster/colony size and the total number of clusters formed in the proportional hazards model demonstrated that only the maximum colony/cluster size was of prognostic significance ($p = 0.0049$ and 0.8442 for colony/cluster size and total number of clusters, respectively).

**Relationship of Growth Pattern in Vitro to Other Prognostic Parameters.** A relationship was sought between the ability of a patient's marrow cells to produce clonal growth in vitro and other factors of known prognostic significance. For the previously untreated patients studied here, age was not of prognostic significance for the outcome of remission induction therapy. The median age for CR patients was 54 years; for RD patients, it was 52 years and, for “other” failures, it was 59 years. In contrast, cytogenetic type was a significant prognostic factor. For the 47 previously untreated patients in whom cytogenetic studies were performed, 20 of 28 NN patients entered CR with 4 failures due to RD and 4 “other” failures. For the 19 patients with NA and AA disease, there were 6 CR, 7 RD, and 6 “other” failures ($p = 0.02$ for the comparison of the percentage of CR for NN versus NA, AA cytogenetic types).

Forty-seven previously untreated patients had both cytogenetic and cloning studies performed on their pretherapy bone marrow aspirates. Logistic regression analysis was performed to determine the simultaneous prognostic significance of both leukemic cell characteristics. When cytogenetic type and total number of cluster/colonies was simultaneously considered, cytogenetic type was a highly significant prognostic factor ($p = 0.006$), and the total number of colonies approached statistical significance ($p = 0.08$). When maximum colony size was substituted for total number of colonies, the prognostic significance of cytogenetic type was unchanged ($p = 0.006$), while the simultaneous prognostic significance of maximal colony size had a p-value of 0.125.

Seven of 9 patients in the best prognostic group (no growth and normal cytogenetics) entered CR, while only 2 of 10 patients whose cells produced >100 clusters or colonies and whose cytogenetics were classified as NA or AA entered CR ($p = 0.03$ for the comparison of the CR rates of these patients versus that of the no growth-normal cytogenetic group) (Table 3). Similarly, only 1 of 9 patients whose cells produced at least one cluster consisting of >20 cells and whose cytogenetic type was NA or AA entered CR ($p = 0.02$ for comparison as above with the best prognostic group). No particular failure type was associated with either poor prognostic category. Too few patients with both adverse in vitro growth characteristics and cytogenetic measurements entered CR for the effects of both characteristics on remission duration to be assessed.

**DISCUSSION**

The in vitro growth characteristics of marrow cells obtained from 166 patients with ANLL are described in this report. There was considerable variability in the growth patterns and in cloning efficiencies of bone marrow cells of ANLL patients in general as well as within each FAB category.

The cloning efficiency of the marrow cells obtained from leukemic patients varied from 0 to 4.5%, with a mean of 0.37 ± 0.5% and a median of 0.06%. The relationship between these values and the true proportion of clonogenic cells in the population is unknown, since the in vitro conditions are likely to be less than optimal and, therefore, a cloning efficiency of less than 100% might simply be a reflection of the inadequacy of growth conditions in vitro.

The studies reported here demonstrated a statistically significant relationship for previously untreated patients between the maximum size of the cluster/colonies formed in vitro and remission duration. The ability of leukemic cells to grow in vitro and the size of the colony/clusters produced is probably reflective of both the ability of the cells to grow under adverse environmental conditions (such as in vitro conditions) and the proliferative potential of the cells. The relationship between these values and the true proportion of clonogenic cells in the population is unknown, since the in vitro conditions are likely to be less than optimal and, therefore, a cloning efficiency of less than 100% might simply be a reflection of the inadequacy of growth conditions in vitro.

While growth pattern in vitro was not statistically significantly related to the outcome of remission induction therapy, assessment of this leukemic cell characteristic together with cytogenetic...
type permitted a distinction to be made between 2 groups of patients, one with a very high likelihood of entering CR with ara-C/anthracycline therapy and another with very little likelihood of entering CR. If further studies with larger numbers of patients confirm these observations, a group of patients will have been identified in whom different remission induction regimens should be tested. Perhaps high-dose ara-C therapy would be effective, since cytogenetic abnormalities do not appear to be of prognostic significance with this therapy (16). Additionally, perhaps the use of recently developed more sophisticated cytogenetic methods would permit a further improvement in the prognostic sensitivity of the studies reported here since, by the recently developed methods, at least one-half of the patients classified as NN by the methods used here would be found to actually belong to the NA or AA group (24).

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Relationship of the Growth of Leukemic Cells in Vitro to the Outcome of Therapy for Acute Nonlymphocytic Leukemia

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