Comparison of Tritiated Thymidine Labeling and Suicide Indices in Acute Nonlymphocytic Leukemia

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ABSTRACT

Leukemic cells were obtained from the bone marrow or peripheral blood of patients with acute myelocytic leukemia or one of its variants. The tritiated thymidine labeling index of the leukemic cells and the suicide index of the cells that produce clusters in vitro were compared. The suicide index was 5 times greater than the labeling index (S.E.) [54 ± 8% versus 12 ± 3%], demonstrating the presence of a highly proliferative subpopulation of leukemic cells (in vitro colony-forming unit) among the relatively slowly proliferating leukemic cells. Since the leukemic in vitro colony-forming unit appears to be the progenitor of the recognizable leukemic cell population, the proliferative characteristics of the recognizable leukemic cells may not be reflective of that of leukemic stem cells.

INTRODUCTION

Acute leukemia in humans is viewed as a disease in which the overall proliferative rate of the leukemic cells is relatively low. This concept is based upon observations of the [3H]dThd2 labeling index, the labeling index of leukemic myeloblasts usually being one-third to one-quarter that of normal myeloblasts (2, 5). Kinetic calculations showed a discrepancy between the apparent size of the leukemic proliferative pool and the rate of growth of the leukemic population. These observations led to the conclusion that either there is ingress of cells from an unrecognizable precursor cell compartment and/or that the nonproliferative cells could resume active proliferation (6, 7). Evidence for the latter has been presented (4, 21), and reentry of the small nonproliferative cells into the cycle has been assumed to account for expansion of the leukemic cell population. The former, however, has not been ruled out as a significant contributing factor.

The introduction of methods to clone normal and leukemic myeloid progenitor cells in vitro in a semisolid matrix has opened the way for studies of morphologically unrecognizable but functionally distinct myeloid progenitor cells, the CFUc (1, 17, 18). The culture of normal marrow in vitro results in the production of both colonies and clusters, whereas acute leukemic marrow cells produce only clusters (13, 22). Cluster production by leukemic marrow is also clonal (14). The studies described here demonstrate that the cell responsible for leukemic cluster formation has a high thymidine suicide index, one that is comparable to that of the normal CFUc.

MATERIALS AND METHODS

Bone marrow specimens were obtained from patients with acute myelocytic leukemia (or one of its variants) or from hematologically normal individuals who had not received chemotherapy for at least 4 weeks. Peripheral blood specimens were obtained from 3 leukemic patients at the same time that their marrow specimens were obtained. The specimens were drawn into a plastic syringe containing 2 ml of 4% sodium citrate for 5 ml of blood or marrow specimens. Erythrocytes were lysed as previously described (20), and the cells were processed as described below.

Mouse marrow was obtained by flushing the contents of 2 femurs into Roswell Park Memorial Institute Medium 1640 made 2% with heat-inactivated fetal calf serum. The cells were washed twice and then processed as described below.

Thymidine Suicide and Labeling Index. A modification of the method of Iscove et al. (9) was used in these studies. Leukemic cells were suspended in Earle’s balanced salt solution at a concentration of 5 × 10⁴ to 5 × 10⁷ cells/ml and incubated for 1 hr at 37° in a humidified atmosphere containing 5% CO₂:95% air. At the end of this period, [3H]dThd (55 Ci/mmol; 0.5 mCi/ml; Schwarz/Mann, Orangeburg, N. Y.) was added to one-half of the cells to a final concentration of 40 μCi/ml. The incubation was continued for 1 hr. The cells were washed 5 times with 50 volumes of cold Earle’s balanced salt solution made 10% with fetal calf serum containing 100 μg of cold thymidine per ml of medium. One-half of the specimen was processed for radioautography as previously reported (20), whereas the other half was plated in agar at 10⁴ cells/plate. Marrow or blood cells not exposed to [3H]dThd were incubated and washed identically to the suicided specimen, except that [3H]dThd was not added to their incubation mixture. These cells served as controls for the suicided specimen. The suicide index was calculated as:

\[
\text{Suicide index} = \frac{\text{no. of control colonies}}{\text{no. of control colonies} - \text{no. of colonies produced by cells incubated with } [3H]dThd}
\]

The proportion of labeled cells (labeling index) was determined by a single individual (H. Preisler). In determining the labeling index, only mononuclear cells were evaluated, and therefore granulocytic forms more mature than myelocytes were excluded from consideration. Hence, in leukemic patients the labeling index reflects the proportion of myeloblasts in S phase, whereas for the normal controls the labeling index represents the proportion of immature cells.

\[\text{No. of control colonies} \times \text{no. of colonies produced by cells incubated with } [3H]dThd\]

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The abbreviations used are: CFUc, in vitro colony-forming unit(s); [3H]dThd, tritiated thymidine.

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(nucleated erythrocytes, myeloblasts, promyelocytes, and myelocytes in S phase).

**Culture of Normal and Leukemic Colony-forming Cells.**

The soft-agar method of Pluznik and Sachs (18) and Bradley and Metcalf (1) was used to culture human cells. A 2.5-ml lower layer of 0.5% agar containing 20% (v/v) conditioned medium was placed into 35-x 10-mm plastic Petri dishes and permitted to solidify at room temperature. Conditioned medium containing human colony-stimulating factor was obtained from a human cell line (3). An upper layer consisting of 0.85 ml of 0.3% agar and containing the 10⁶ cells to be cultured was layered on top of the 0.5% agar layer. The agar was permitted to solidify at room temperature and was then inspected for cell clumps (none were noted in the experiments reported here).

Cultures were set up in quintuplicate and incubated at 37° in a humidified incubator in an atmosphere consisting of 5% CO₂:95% air. Groups consisting of ≥20 cells were counted as colonies, whereas groups consisting of lesser numbers of cells were considered to be clusters (5). For mouse marrow a colony was defined as consisting of >40 cells. On Day 7 of the culture 10 clusters were picked from the plates of each patient for cytological study. The cells were smeared on a slide and stained with Wright-Giemsa. The plates were incubated for a further 4 to 7 days at which time growth was reevaluated.

Mouse bone marrow was cultured at a concentration of 2 x 10⁶ cells/plate in the plasma clot system of McLeod et al. (11) as previously described (16).

**RESULTS**

Initially, suicide studies were carried out on 6 normal mouse bone marrow specimen and 6 normal human marrow specimens. [³H]dThd labeling indices were simultaneously determined on 3 each of the mouse and human specimens. Table 1 gives the results of these studies. The mean suicide index for murine CFUc was 46 ± 5% (S.E.), with a labeling index of 24 ± 4%. For normal human marrow the mean suicide index was 29 ± 4%, with a labeling index of 19 ± 4%.

Studies were carried out on bone marrow specimens obtained from 12 patients with acute myelocytic leukemia

<table>
<thead>
<tr>
<th>Specimen</th>
<th>% of mouse marrow</th>
<th>% of human marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suicide index</td>
<td>Labeling index</td>
<td>Suicide index</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>ND</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>46 ± 5</td>
<td>24 ± 4</td>
</tr>
</tbody>
</table>

a ND, not determined.

b Mean ± S.D.

The studies reported here demonstrate that the [³H]dThd suicide index for leukemic CFUc was significantly greater than the labeling index of the leukemic cells among which the CFUc were found. Hence, the proportion of leukemic cells that were in S phase as measured by the labeling index was not reflective of the proportion of leukemic CFUc that were synthesizing DNA. The suicide index of the leukemic CFUc was similar to that reported previously for the CFUc of normal marrow (9, 15) and somewhat greater than the suicide index of the CFUc of 6 hematologically normal individuals reported in this paper. There appeared to be some tendency for the labeling index and the suicide index to vary in the same direction. The labeling index and suicide index of the marrow cells of Patient 8 declined during therapy. Similarly, the labeling index of the marrow leukemic cells of 2 (of 4) additional patients was very low (<1%) 24 hr after the initiation of therapy, and there was no demonstrable suicide of these patients' CFUc. The suicide index of the 3 other patients' CFUc was high (40 and 95%)

**DISCUSSION**

The studies reported here demonstrate that the [³H]dThd suicide index for leukemic CFUc was significantly greater than the labeling index of the leukemic cells among which the CFUc were found. Hence, the proportion of leukemic cells that were in S phase as measured by the labeling index was not reflective of the proportion of leukemic CFUc that were synthesizing DNA. The suicide index of the leukemic CFUc was similar to that reported previously for the CFUc of normal marrow (9, 15) and somewhat greater than the suicide index of the CFUc of 6 hematologically normal individuals reported in this paper. There appeared to be some tendency for the labeling index and the suicide index to vary in the same direction. The labeling index and suicide index of the marrow cells of Patient 8 declined during therapy. Similarly, the labeling index of the marrow leukemic cells of 2 (of 4) additional patients was very low (<1%) 24 hr after the initiation of therapy, and there was no demonstrable suicide of these patients' CFUc. The suicide index of the 3 other patients' CFUc was high (40 and 95%)
Table 2

Comparison of $[^3H]dThd$ labeling index and CFUc suicide index of leukemic cells

Patients 2 to 4, 7, 8, 11, and 12 were previously untreated patients, whereas the remainder of the patients were in their first or second relapse. Patients 9 to 12 were studied 24 hr after the initiation of chemotherapy (9).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>% of myeloblasts and promyeloblasts</th>
<th>Cluster growth</th>
<th>Suicidin index (%))</th>
<th>Labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AML</td>
<td>74</td>
<td>36 ± 4</td>
<td>23 ± 2</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>AMMoL</td>
<td>58</td>
<td>37 ± 10</td>
<td>7 ± 2</td>
<td>81</td>
</tr>
<tr>
<td>3</td>
<td>AProL</td>
<td>71</td>
<td>65 ± 8</td>
<td>47 ± 5</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>AEL</td>
<td>62</td>
<td>49 ± 9</td>
<td>31 ± 2</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>AEL</td>
<td>94</td>
<td>377 ± 43</td>
<td>165 ± 17</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>AProL</td>
<td>72</td>
<td>27 ± 6</td>
<td>11 ± 6</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>AMMoL</td>
<td>82</td>
<td>16 ± 1</td>
<td>8 ± 1</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>AMMoL</td>
<td>91</td>
<td>194 ± 8</td>
<td>48 ± 4</td>
<td>75</td>
</tr>
<tr>
<td>9</td>
<td>AEL</td>
<td>91</td>
<td>53 ± 9</td>
<td>31 ± 7</td>
<td>42</td>
</tr>
<tr>
<td>10</td>
<td>AMMoL</td>
<td>28</td>
<td>15 ± 1</td>
<td>9 ± 1</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>AML</td>
<td>58</td>
<td>11 ± 3</td>
<td>0.6 ± 0.4</td>
<td>95</td>
</tr>
<tr>
<td>12</td>
<td>AML</td>
<td>97</td>
<td>11 ± 3</td>
<td>13 ± 1</td>
<td>0</td>
</tr>
</tbody>
</table>

a AML, acute myelocytic leukemia; AMMoL, acute myelomonocytic leukemia; AProL, acute promyelocytic leukemia; AEL, acute erythroleukemia.
b Mean ± S.D.
c Percentage of leukemic cells in S phase, estimated from a DNA histogram by the method of Krishan (10).
d Specimens obtained prior to (a) and 24 hr after (b) the initiation of therapy.

Table 3

Comparison of thymidine labeling indices and CFUc suicide indices of acute myelocytic leukemic cells obtained from the bone marrow and peripheral blood

The patients correspond to those in Table 2.

<table>
<thead>
<tr>
<th>Patient</th>
<th>% of bone marrow</th>
<th>% of peripheral blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblasts</td>
<td>Suicide index</td>
<td>Labeling index</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>81</td>
</tr>
<tr>
<td>5</td>
<td>94</td>
<td>56</td>
</tr>
<tr>
<td>8</td>
<td>91</td>
<td>75</td>
</tr>
</tbody>
</table>

after 24 hr of therapy, and the labeling indices were 7 and 10%, respectively.

These observations do not agree with those of the single reported study of the tritiated suicide index of the CFUc of acute myelocytic leukemia (15). In this study the pooled suicide index of leukemic CFUc was reported to be 17 ± 10%. The fact that the suicide index was substantially lower than that of the CFUc of normal bone marrow led to the conclusion that the rate of proliferation of leukemic CFUc was less than that of normal CFUc, much as the labeling index of leukemic myeloblasts is less than that of normal myeloblasts (12). These authors did not simultaneously determine the labeling index of the leukemic cells so that a comparison of the labeling index and suicide index for their patients cannot be made.

Four methodological differences exist between our study and the previously reported study. In our suicide studies $[^3H]dThd$ was used at a higher specific activity (54 versus 23 Ci/mmol), at a higher concentration (40 versus 20 µCi/ml), and for a longer time (60 versus 30 min). In addition, we preincubated the leukemic cells for 60 min prior to the addition of $[^3H]dThd$. These methodological differences by themselves, however, do not appear to account for the differences in observations since the suicide index of both normal human and mouse marrow reported in the present paper is comparable to (and actually slightly less for human marrow) that reported in the literature (9, 15).

The reasons for the differences in the suicide index of leukemic CFUc in the previous report compared to the alluded to report are not clear. The previous authors studied only 9 specimens, some of which were derived from peripheral blood. The labeling indices of circulating leukemic cells are approximately one-third that of marrow cells (2, 5). In the 3 matched specimens that we studied, the mean suicide index for bone marrow CFUc was 71 ± 8%, whereas for peripheral blood CFUc it was 20 ± 8%. Hence, the pooling of data derived from marrow and peripheral blood may have lowered the mean suicide index. Unfortunately, the data on the labeling indices of the leukemic cells in the study referred to were not reported. Another possible explanation relates to our use of a preincubation of 1 hr at 37°. Perhaps leukemic CFUc are more susceptible to metabolic derangements upon removal from the body (e.g., a fall in energy stores) than are normal CFUc and require a preincubation period at 37° to resume whatever phase of the cell cycle that they were in while in vivo. Clearly, further studies.
will be required to clarify this issue.

If the relationship between the leukemic CFUc and the recognizable leukemic myeloblasts is similar to that which exists between the normal CFUc and normal myeloblasts, the leukemic CFUc may be viewed as a precursor to the morphologically recognizable leukemic cells. This possibility is reinforced by the fact that the suicide index of the leukemic CFUc is similar in magnitude to the labeling index of the large proliferative leukemic myeloblasts (6). If this were the case, progression of the large proliferative myeloblasts to smaller quiescent myeloblasts could represent the completion of a maturation sequence. Consequently, the role of the small quiescent cells as a progenitor pool for the large proliferating cells would have to be reevaluated. Any firm conclusion relating to this issue cannot as yet be made. Although the number of CFUc may appear to be too low to sustain the recognizable leukemic cells (only 1 cell: 1,000 to 10,000) specific calculations cannot be made because neither the seeding efficiency nor the cycle time of the CFUc is known. Similarly, the earlier observations that quiescent cells can apparently resume DNA synthesis cannot be taken as proof for the functioning of these cells as a progenitor population for the large proliferating leukemic blasts since the number of cell cycles that these reentering cells can go through is not known. The observations reported in this paper of the existence of a rapidly proliferating clonogenic population of leukemic cells reopen the question as to the nature of the leukemic precursor population that sustains the recognizable leukemic cells.

Finally, there are contradictory reports in the literature about the relationship between the leukemic cell labeling index and the response to remission induction therapy during the treatment of acute leukemia (8, 23). The demonstration of the discordance between the labeling index of the leukemic cells and the suicide index of the leukemic CFUc puts this dispute into sharper focus. If the proliferative characteristics of the leukemic CFUc more closely reflects that of the leukemic stem cells than does the labeling index, the relationship between the latter and response to therapy may have been fortuitous in the few studies in which such a relationship was found. In any event it would seem to be rather simplistic to expect a clear-cut relationship between any pretherapy cell cycle kinetic measurement and clinical response to therapy since the metabolic sensitivity of the leukemic cell to the chemotherapeutic agents used is not taken into account when one considers only the kinetic parameters. Perhaps determination of the effect of 24 hr of therapy upon the number of leukemic CFUc as well as their suicide index will correlate with therapeutic response and permit 24-hr trials of chemotherapy before a patient is committed to a full course of intensive chemotherapy.

REFERENCES


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