Cellular Maturation in Human Preleukemia

By H. Phillip Koeffler and David W. Golde

Bone marrow cells from three preleukemic patients with prominent marrow karyotypic abnormalities were studied in liquid culture to determine if the neoplastic clones were capable of maturation. Parallel cytogenetic and cytologic studies were performed in sequentially harvested bone marrow cultures. Maturation, albeit delayed, occurred in cultures from all three patients. By 14 days of culture in vitro, morphologic, cytochemical, and functional evidence of maturation was observed in about 70% of the cells. By day 21, 85% of the cells were mature by these criteria. All but 2 of 249 metaphases from the cultured cells contained the cytogenetic abnormality of the neoplastic clone. We conclude that some preleukemic cells identified by a chromosomal abnormality can mature in vitro. Preleukemia may be viewed as a syndrome of "early leukemia" in which the neoplastic clone is established and manifested functionally as ineffective hematopoiesis. Hematopoietic cell differentiation becomes progressively abnormal with termination in the nearly complete maturational block characteristic of acute myelogenous leukemia.

Preleukemia is a recognizable clinical syndrome of hematopoietic dysfunction preceding the typical findings of acute myelogenous leukemia. The preleukemic syndrome is generally characterized by peripheral blood cytopenia and normal or hypercellular bone marrow with morphologic abnormalities in cell differentiation. Culture studies in vitro of bone marrow cells in preleukemia have shown decreased or absent granulocyte-monocyte colony formation, defects in cell maturation, and abnormalities in the production of colony-stimulating activity (CSA).

Acute myelogenous leukemia is thought to arise from neoplastic transformation at the pluripotent stem cell level leading to a block in cell maturation at the recognizable myeloblast or promyelocyte stage. A central question in the study of acute myelogenous leukemia is whether the leukemic cells are capable of maturation under certain environmental circumstances or whether the defect is complete and unchangeable by external conditions. Although this question may be of profound therapeutic importance, a clear answer has not emerged from numerous studies. Culture in vitro of hematopoietic cells has been used extensively to study the problem, but conflicting results have been reported. In the present study we attempted to determine if preleukemia myeloid cells are capable of maturation in vitro. In order to be certain we were monitoring cells of the neoplastic clone, we selected patients with a prominent chromosomal abnormality so that sequential cytogenetic analysis could conveniently be performed on a relatively large number of cells.
Table 1. Clinical Characteristics of Preleukemia Patients Studied

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)/Sex</th>
<th>Palpable Spleen</th>
<th>WBC Count</th>
<th>Hemoglobin</th>
<th>Platelet Count</th>
<th>LAP</th>
<th>Bone Marrow Cellularity</th>
<th>Cytogenetic Abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>78/M</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
<td>N</td>
<td>↑</td>
<td>47, XY, + 8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25/F</td>
<td>+</td>
<td>N</td>
<td>↓</td>
<td>N</td>
<td>↑</td>
<td>45, XX, - C</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>79/M</td>
<td>-</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>47, XY, + 9</td>
<td></td>
</tr>
</tbody>
</table>

N, normal; ↑, increased; ↓, decreased; +, present; −, absent; LAP, leukocyte alkaline phosphatase.

MATERIALS AND METHODS

Patients. Three preleukemic patients with stable aneuploidy were studied (Table 1). The patients had blood and bone marrow abnormalities typical of preleukemia as described in the literature.

All were anemic with low corrected reticulocyte counts, and oval macrocytosis was prominent. Patients 1 and 2 required regular red blood cell transfusions. All patients were thrombocytopenic. Their bone marrow at the time of study was hypercellular with prominent erythroid abnormalities. The red cell precursors were megaloblastoid, and although myelopoiesis was dysplastic the abnormalities were not sufficient to diagnose acute myelogenous leukemia. The patients' bone marrow was "left shifted," however, there were less than 10% blast forms present in the marrow smears. The chromosomal abnormalities were trisomy 8, trisomy 9, and C-group monosomy. Patients 1-3 developed acute myelogenous leukemia 5, 3, and 6 mo respectively, after study and retained the same karyotypic abnormality.

Twenty healthy volunteers were used as controls.

Cell culture. Bone marrow was obtained from the patients and appropriately informed healthy volunteers by aspiration from the iliac crest into a heparinized syringe. The nucleated cells were isolated by sedimentation and centrifugation and resuspended in tissue culture media. Three million nucleated marrow cells were cultured in liquid suspension using the Marbrook diffusion chamber in vitro as previously described. Cultures were harvested at intervals up to 28 days for viable and differential cell counts. Cytocentrifuge preparations were made and stained using the following methods: Giemsa, periodic acid Schiff (PAS), ASD chloroacetate esterase, and α-naphthyl butyrate esterase. These special stains helped in determining cell lineage. For example, chloroacetate esterase was taken as a marker of granulocytes, while α-naphthyl butyrate was used to identify cells of the monocyte-macrophage series. Morphologic classification of cellular maturation in the granulocytic and monocytic series was as follows: Immature cells were taken to include only blasts, progranulocytes, and promonocytes; mature cells included macrophages, myelocytes, polymorphonuclear neutrophils, eosinophils, and red cell precursors.

Phagocytosis was assessed using yeast cells. Tubes containing 0.1 ml cell suspension (5 × 10⁶ cells), 0.1 ml Hanks' balanced salt solution (HBSS) containing 10% heat-inactivated fetal
calf serum (FCS), and 0.5 ml Candida pseudotropicalis (1 x 10^6 yeast) were incubated on a rocker panel at 37°C. After 30 min cytocentrifuge preparations were made and stained with Giemsa. Only Candida completely surrounded by monocyte or granulocyte cytoplasm were considered phagocytized. The number of yeast ingested by 200 cells was counted and the mean number of phagocytized yeast per cell calculated.

Cytogenetics. Cytogenetic studies were performed on fresh bone marrow, fresh peripheral blood cells, and cells harvested at different time periods from liquid culture. Cultures were prepared for cytogenetic analysis by adding colcemid (0.02 μg/ml), and were then processed by routine methods. A 72-hr whole blood culture with phytohemagglutinin (PHA) was used to study lymphocyte chromosomes. Standard air-dried chromosome preparations were made and stained with Giemsa, and the metaphases were examined. Approximately 15-30 metaphases were analyzed from each culture when possible. Giemsa-banding analysis was performed once on bone marrow specimens from the three patients in order to define the precise karyotypic abnormality.

RESULTS

Growth in liquid culture. Viable cell counts in liquid culture over the period studied were within or slightly above the normal range established for 20 healthy volunteers (Fig. 1). This pattern of cell growth was similar to previously studied preleukemic patients. After 2 wk in culture, viable cell counts ranged between 1 and 1.6 x 10^6.

The patterns of cellular maturation in vitro are shown in Fig. 2 and Table 2. During 7 days in culture, the percentage of immature cells increased markedly. By 7 days 40%-55% of the cells were immature. Maturation, albeit delayed, occurred among all the preleukemic cell populations. By day 14, 65%-75% of

<table>
<thead>
<tr>
<th>Culture (Days)</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Immature Cells</td>
<td>Mature Cells</td>
<td>Immature Cells</td>
</tr>
<tr>
<td>0</td>
<td>4.5 x 10^5</td>
<td>25.5 x 10^5</td>
<td>6.2 x 10^5</td>
</tr>
<tr>
<td>4</td>
<td>13.0 x 10^5</td>
<td>12.0 x 10^5</td>
<td>5.5 x 10^5</td>
</tr>
<tr>
<td>7</td>
<td>8.3 x 10^5</td>
<td>6.8 x 10^5</td>
<td>5.7 x 10^5</td>
</tr>
<tr>
<td>10</td>
<td>8.0 x 10^5</td>
<td>12.0 x 10^5</td>
<td>3.5 x 10^5</td>
</tr>
<tr>
<td>14</td>
<td>6.0 x 10^5</td>
<td>10.0 x 10^5</td>
<td>3.0 x 10^5</td>
</tr>
<tr>
<td>21</td>
<td>1.7 x 10^5</td>
<td>9.4 x 10^5</td>
<td>0.7 x 10^5</td>
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</table>
the cells were mature; by day 21, 85%; and by day 28, 95% of the cells were morphologically mature (Fig. 3). The preleukemic cells harvested from liquid culture on day 14 phagocytized *C. pseudotropicalis* in a manner similar to normal cells; 60% of the morphologically mature cells phagocytized one or more yeast cells, and a mean of 1.2 yeast cells were ingested per morphologically mature cell.

**Cytogenetic studies.** Table 3 summarizes the results of the cytogenetic studies

<table>
<thead>
<tr>
<th>Patient</th>
<th>Peripheral Blood (PHA)</th>
<th>Fresh Marrow</th>
<th>Cultured Marrow</th>
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<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 7</td>
<td>Day 10</td>
</tr>
<tr>
<td>1</td>
<td>46, XY (15)</td>
<td>47, XY, + B (57)</td>
<td>47, XY, + B (59)</td>
</tr>
<tr>
<td>2</td>
<td>46, XX (15)</td>
<td>45, XX, − C (15)</td>
<td>45, XX, − C (14)</td>
</tr>
<tr>
<td>3</td>
<td>46, XY (15)</td>
<td>47, XX (1)</td>
<td>46, XX (1)</td>
</tr>
</tbody>
</table>

Parentheses, number of metaphases examined.
on fresh bone marrow, PHA-stimulated peripheral blood lymphocytes, and cultured marrow cells. Patient 1 was studied on three separate occasions; because the same karyotype was obtained in each study these data are combined. In each patient all of the peripheral blood lymphocytes responding to PHA had a normal karyotype. All of the fresh and cultured bone marrow metaphases from patients 1 and 3 showed trisomy of chromosomes 8 and 9, respectively, in a total of 294 metaphases examined. All fresh and cultured marrow metaphases (81) from patient 2 were hypodiploid 45,XX,-C karyotype, except for a single normal 46,XX metaphase from days 7 and 14 of liquid culture.

**DISCUSSION**

The pathogenesis of preleukemia, like that of acute myelogenous leukemia, is unknown. There is good evidence that acute leukemia is a clonal disorder involving hematopoietic stem cells.9,11 The most prominent cellular abnormality in acute leukemia is a maturation block at the myeloblast-monoblast stage of development. The accumulation of leukemic blast cells is due not to their rapid rate of proliferation but rather to their inability to mature to end cells and be removed.11,26 Cytogenetic studies in preleukemia suggest that in most patients with karyotypic abnormalities an identifiable neoplastic clone is established.2,27 During the preleukemia phase, however, ineffective hematopoiesis is the prominent abnormality, rather than an accumulation and overgrowth of blast cells. These considerations can be reconciled by hypothesizing that during the preleukemic state some cells are capable of maturation, and cell death may therefore occur.

We approached the question of cell maturation in preleukemia by culturing bone marrow cells in liquid medium and doing sequential and parallel cytogenetic and cytologic studies. The three preleukemic patients were selected because they had typical disease and had a stable aneuploid chromosomal abnormality. Two patients had trisomy and one monosomy, thereby allowing for convenient identification of the neoplastic metaphases. Although the previously observed pattern of delayed maturation in liquid culture was seen, maturation did proceed progressively with time. By 14 days of culture, morphologic, cytochemical, and functional evidence of maturation was observed in about 70% of the cells. By day 21, 85% of the cells were mature.

Total viable cell counts fell during the culture period, and maintenance of cell numbers representing 50% of the initial inoculum required cellular proliferation.7,28 In order to reasonably exclude the possibility that the mature cells observed later in culture were derived from a normal clone, we did sequential cytogenetic analyses on a relatively large number of metaphases. Only two metaphases in one of the patients studied were found to be normal. The remaining metaphases (247 analyzed) carried the cytogenetic abnormality of the neoplastic clones. Mature granulocytes remain viable in liquid culture for less than 48 hr; thus, the mature cells seen later in culture must have been derived from replicating precursors. Because almost all these precursors were of the neoplastic clone, we conclude that some of the abnormal hematopoietic cells from these patients with preleukemia were capable of maturation in vitro. Likewise, since all the dividing hematopoietic cells from the fresh marrow aspirate
contained the chromosomal abnormality, it is likely that most of the patients’ mature circulating blood cells were also progeny of the abnormal clone. The possibility remains, however, that the patients’ circulating mature cells originated from a normal clone of marrow precursors not detected by the cytogenetic analysis of fresh marrow preparations.

Extensive studies in vitro of cell differentiation in human acute myelogenous leukemia have provided conflicting data. Some authors have claimed that normally differentiated colonies grown in semisolid gel culture from leukemic patients represented leukemic cell maturation. Others, however, have reported that leukemic colonies showed little evidence of cellular differentiation. The difficulty in interpreting reports of leukemic cell maturation in vitro relate to the possibility that the normally differentiated colonies arise from residual nonleukemic precursors. This problem is of major concern because low cloning efficiencies are usually observed in solid gel cultures. Without a cytogenetic or enzymatic marker, it is not possible to be certain of the origin of a mature cell. A similar problem exists in interpreting the conflicting data on human leukemic cell differentiation in diffusion chambers implanted into the peritoneal cavity of mice. Abnormal leukemic blast cell maturation leading to cell death in Marbrook chambers has been reported in one patient with AML. Most AML cells, however, do not show this pattern. Presently, the weight of evidence suggests that acute myelogenous leukemia cells are not usually capable of substantial maturation in vitro. In certain murine leukemias, however, cell differentiation may clearly be induced in culture.

Our observations suggest that neoplastic preleukemic cells can mature in vitro. While cell differentiation was less than normal, it was clearly greater than that seen in acute myelogenous leukemia. Preleukemia may be viewed as an “early” leukemic syndrome in which hematopoietic cell differentiation becomes progressively impaired with termination in the nearly complete maturation block characteristic of acute myelogenous leukemia.

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REFERENCES
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