Accelerated Myeloblast Destruction and Abnormal Lysosome Disruption in Cultured Bone Marrow: Association With Indolent Acute Myelogenous Leukemia

By Richard F. Branda, Harry S. Jacob, Steven D. Douglas, Charles F. Moldow, and Ricard R. Puumala

Despite no chemotherapy and a marrow morphologically typical of frank relapse, an acute myelogenous leukemia (AML) patient survived for nearly 1 yr. During this time she remained asymptomatic and maintained nearly normal levels of platelets and hemoglobin. Cytochemical and electron microscopic studies of her bone marrow in liquid culture revealed on several occasions a unique maturational sequence in that leukemic cells differentiated to form morphologically abnormal primary granules which appeared to rupture and cause cytolysis of these cells. In these cultures, blasts rapidly disappeared and were replaced by more mature granulocytes, in contrast to observations in cultures derived from five other patients with AML in relapse which showed persistently elevated blast counts with no evidence of maturation in vitro. These findings support the concept that in AML cell maturation is regularly impaired and in some cases also aberrant. In addition, the abnormal granule formation with autolysis of the leukemic cells observed in one patient may explain both the early cell death in vitro and this patient’s relatively indolent clinical course. Similar in vitro studies may help predict atypical clinical courses in patients with AML and facilitate design of appropriate chemotherapy.

Survival of patients with acute myelogenous leukemia (AML) usually correlates with remission duration. Rare patients, however, develop a poorly understood apparent symbiosis with their disease and have relatively prolonged survival, despite persistence of leukemic cells. We have recently observed such a patient, who, without treatment, lived asymptptomatically for more than a year after relapse of AML. Cytochemical and electron microscopic studies of her bone marrow cells in culture have revealed an unusual maturational abnormality which, we reason, was related to the slow progression of her disease.

CASE REPORT

R.G., a 26-yr-old white woman, noted excessive fatigue, petechiae, and gingival bleeding 1 mo after delivery of her third child. Evaluation revealed fever, petechiae, ecchymoses, splenomegaly.
and mastitis. The hemoglobin was 8.6 g/100 ml, white count 15,500/cu mm with 94% blasts, platelet count 18,000/cu mm, and serum lysozyme 24.6 μg/ml (normal range 2.0–11.9 μg/ml). A bone marrow aspirate showed 71% myeloblasts containing peroxidase positive granules, frequent Auer rods, and vacuoles (see Fig. 3A); bone marrow sections were hypercellular. She was treated with appropriate antibiotics and was begun on chemotherapy with cyclophosphamide, methotrexate, and cytosine arabinoside.

After two cycles of chemotherapy, her marrow became markedly hypocellular with less than 5% blasts, and within 2 wk assumed normal cellularity. Monthly cyclophosphamide, methotrexate, and cytosine arabinoside as maintenance therapy was begun, and her bone marrow remained normocellular with less than 5% blasts for 5 mo, when blasts with Auer rods reappeared. The patient refused further chemotherapy for religious reasons, despite several efforts to convince her otherwise.

Over the next 4 months she developed gradually progressive anemia, thrombocytopenia, and leukocytosis and seemed to be in typical clinical relapse. However, in the course of a severe streptococcal infection of the face and ear, treated with antibiotics and prednisone, 40 mg/day, the peripheral blast count strikingly declined. A white count of 40,800/cu mm with 97% blasts dropped precipitously to 1100/cu mm. During the following 7 mo, the white count remained less than 10,000/cu mm, with reasonable granulocyte numbers, despite the fact that bone marrows continued predominantly blastic—a dichotomy suggesting blast destruction in the marrow. For example, a bone marrow examination done 8 mo after the onset of overt relapse showed 46% myeloblasts (large cells [15-20 μ] with a high nucleocytoplasmic ratio, fine nuclear chromatin pattern, prominent nucleoli, basophilic cytoplasm, few azurophilic granulations, frequent Auer rods, and prominent vacuolization). One per cent of the cells contained numerous peroxidase-positive granules, abundant cytoplasm, and large eccentric nuclei with nucleoli; these promyelocytes occasionally contained Auer rods and vacuoles (Fig. 3A). Neutrophilic myelocytes, band forms, and polymorphonuclear leukocytes represented 27.4% of 1000 cells counted. The differential distribution of other cell types was: red cell precursors, 19%; eosinophils, 1.6%; lymphocytes, 5.0%.

Eleven months after first evidence of relapse, and 16 mo after diagnosis, she again developed progressive anemia, thrombocytopenia, and leukocytosis. One month later massive hemorrhage led to her death.

**MATERIALS AND METHODS**

**Cell Culture**

Marrow aspirates were obtained by iliac puncture with the informed consent of the patient; 3–5 ml of marrow mixed with 500 units of heparin were passed through progressively smaller gauge needles (Nos. 18–25). After addition of an equal volume of CMRL 1066 (Grand Island Biological Co., Grand Island, N.Y.), pH 7.8, containing 1%, glutamine, 15% fetal calf serum (Reheis Chemical Co., Kankakee, Ill.), and 1% penicillin-streptomycin (Grand Island Biological Co.), the mixture was layered onto a Ficoll–Hypaque gradient and centrifuged at 400 g for 30 min at 4°C to remove mature red cells and polymorphonuclear leukocytes. Preliminary experiments indicated that removal of these mature cells did not alter the in vitro growth characteristics of bone marrow cells and greatly facilitated morphological observations. Cells at the interface were collected, washed three times with media, counted, and tested for viability by trypan blue exclusion. Cell concentration was adjusted to approximately 2 x 10^6 viable cells/ml; 1 ml was placed in a modified Marbrook diffusion chamber flask. This system consisted of a glass bulb closed at one end by dialysis membrane and suspended in a 125-ml Erlenmeyer flask containing approximately 75 cc of media. The flasks were incubated at 37°C with humidity and 5% carbon dioxide.

The cells were harvested at intervals by flushing the chambers with culture medium. Viable cells (trypan blue) were counted and slides prepared by Cytospin (Shandon, Inc., London, England). An aliquot was fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate for electron microscopy. Specimens were postfixed in osmium tetroxide, dehydrated, and embedded in epon. They were en bloc stained with uranyl acetate, sections stained with lead citrate and examined in a Siemens 102 electron microscope. Appropriate slides were stained for myeloperoxidase, alkaline phosphatase, with toluidine blue and Wright's stain.
Viable cell counts decreased progressively when marrow from 26 control (nonhematologic malignancy) patients were cultured (stippled area, Fig. 1A), and this decremental rate was no different in cultures from five other patients with AML in relapse (triangles, Fig. 1A). In contrast, cultures of the relapse marrow from patient R.G. underwent a striking and significantly greater decrease in viable cells, especially during the first few days of culture (circles, Fig. 1A). At the time of this marrow examination, the patient had been in frank relapse for 8 mo and had received no chemotherapy during that period. Conversely, cell counts in three separate cultures obtained during remission from R.G. were not significantly different from those obtained from cultures of 12 other patients with AML in remission, or from cultures from the 26 patients with nonmalignant conditions (Fig. 1B).

When differential counts were performed from cultures of R.G. and compared to those from other AML patients in relapse, differences were also noted.

Patients

Bone marrow aspirates for culture were obtained from patient R.G. at the time of diagnosis, twice during remission, early in relapse, and 8 mo after the onset of overt relapse. Proliferation and maturation of these cells in culture were compared with similar cultures from five other patients with AML in relapse, 12 patients in complete remission of AML, and 26 patients with nonmalignant hematologic conditions.

RESULTS

Viable cell counts decreased progressively when marrow from 26 control (nonhematologic malignancy) patients were cultured (stippled area, Fig. 1A), and this decremental rate was no different in cultures from five other patients with AML in relapse (triangles, Fig. 1A). In contrast, cultures of the relapse marrow from patient R.G. underwent a striking and significantly greater decrease in viable cells, especially during the first few days of culture (circles, Fig. 1A). At the time of this marrow examination, the patient had been in frank relapse for 8 mo and had received no chemotherapy during that period. Conversely, cell counts in three separate cultures obtained during remission from R.G. were not significantly different from those obtained from cultures of 12 other patients with AML in remission, or from cultures from the 26 patients with nonmalignant conditions (Fig. 1B).
In cultures from the five other patients, initially high blast counts remained elevated for more than 1 wk (stippled area, Fig. 2A); moreover, little morphological change in the appearance of the cells and no granule formation were detected. Concomitantly, mature neutrophils declined progressively (stippled area, Fig. 2B), and macrophages became the predominant cell type after about 10 days. In striking contrast, bone marrow cells cultured from R.G. during relapse manifested a rapid drop in blasts—cells with a high nucleocytoplasmic ratio, prominent nucleoli, and no or few cytoplasmic granules (Fig. 2A) and a concomitant increase in more mature, granulated cells. The appearance of numerous cytoplasmic granules, with cytochemical characteristics of azurophilic granules, was considered evidence of cellular maturation. (These cells were counted as "granulocytes" [Fig. 2B].) Subsequently, these more differentiated cells also declined at a time when total viable cell numbers were also decreasing (Fig. 1A).

Cultures of nonmalignant bone marrows showed no evidence of abnormal maturation (Table 1). Blasts and granulocyte precursors (leukocytes with no or few azurophilic granules) decreased progressively due to maturation or cell death. Similarly, more mature neutrophils (cells with neutrophil-specific granules), the predominant cell type on day 0, declined throughout the incubation period. In contrast, macrophages rapidly increased in number, becoming the predominant cell type after about 1 wk in culture. The percentage of lymphocytes and eosinophils remained stable throughout the culture period. Most erythrocyte precursors were lost in the preparative procedure. Similar

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Table 1. Differential Cell Counts in Cultures of Bone Marrow From 12 Patients
With Nonmalignant Hematologic Conditions*

<table>
<thead>
<tr>
<th>Day</th>
<th>Blasts (% ± SD)</th>
<th>Neutrophils (% ± SD)</th>
<th>Macrophages (% ± SD)</th>
<th>Lymphocytes (% ± SD)</th>
<th>Eosinophils (% ± SD)</th>
<th>Red Cell Precursors (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.4 ± 1.2</td>
<td>76.5 ± 14.2</td>
<td>0</td>
<td>24 ± 13.4</td>
<td>3.1 ± 2.1</td>
<td>4.6 ± 5.9</td>
</tr>
<tr>
<td>4</td>
<td>0.5 ± 0.3</td>
<td>67.5 ± 21.5</td>
<td>8.0 ± 7.6</td>
<td>22.4 ± 20.8</td>
<td>1.3 ± 1.0</td>
<td>0.7 ± 0.8</td>
</tr>
<tr>
<td>8</td>
<td>0.3 ± 0.4</td>
<td>33.3 ± 28.2</td>
<td>43.4 ± 27.6</td>
<td>25.1 ± 24.9</td>
<td>6.3 ± 6.7</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>26.8 ± 15.8</td>
<td>68.8 ± 26.3</td>
<td>15 ± 15.4</td>
<td>3.6 ± 2.9</td>
<td>0</td>
</tr>
</tbody>
</table>

* Differential counts were performed on Wright-Giemsa-stained smears; 200 cells were counted.
Fig. 3. Photomicrographs of bone marrow cultures from patient R.G. (A–D) Marrow obtained at presentation; (E–G) marrow obtained during relapse. (A) Presentation bone marrow showing Auer rods, vacuolization, and granule formation in immature granulocytes. (B) After 4 days of culture, showing numerous large granules in almost every cell. (C) After 7 days of culture, showing persistence of large granules in cells with a decreased nucleocytoplasmic ratio. (D) After 12 days of culture—predominantly macrophages. (E) Cells from relapse culture on day 1 showing abnormal granule formation. (F) After 3 days of culture, showing extensive vacuolization. (G) After 8 days of culture, showing predominantly macrophages. x 1440.
changes in cellular composition were seen in cultures of bone marrows from patients in the remission period of AML.

Morphologically, direct smears of the marrow at the time of diagnosis and prior to treatment consisted predominantly of blast cells (71%), many with Auer rods and vacuoles; a few malignant cells appeared to be forming granules (Fig. 3A). After 4 days in culture (Fig. 3B), nearly every immature cell had developed peroxidase-positive granules, but these granules were larger and more numerous than in normal promyelocytes. After 7 days (Fig. 3C), the granulated cells were larger and showed a decreased nucleocytoplasmic ratio, but the nuclei retained characteristics of immaturity, with a fine chromatin pattern and prominent nucleoli. After 12 days (Fig. 3D), normal-appearing macrophages became predominant, but an occasional cell with granules could be found. Following chemotherapy-induced remission, two bone marrow cultures showed a maturational sequence similar to that in cultures of nonmalignant conditions and of other patients with AML in remission.

Eight months later, when the patient was in frank morphological relapse with 46%, blasts in the marrow, but virtually normal peripheral counts and morphology, marrow cultures were particularly aberrant. Abnormal granule formation was again seen in leukemic cells, but these cells were now much more bizarre in appearance. Thus, after 1 day in culture most of the blasts had developed extremely large and clearly abnormal granules (Fig. 3E). Moreover, by day 3 most of these granules appeared to have lysed spontaneously with accompanying prominent cellular vacuolization and death (Fig. 3F). One day later (day 4), extensive autolysis of the malignant cells was evident (not shown). After 8 days, macrophages were again the predominant cell type (Fig. 3G). The abnormal granules were myeloperoxidase positive, metachromatic with toluidine blue, and negative for alkaline phosphatase—all characteristics of primary granules.

Electron microscopic studies of these malignant cells after 1 day in culture showed a predominance of immature blast-like cells with euchromatic nuclei, large Golgi zones, and well developed rough-surfaced endoplasmic reticulum; these cells contained a single granule type which varied in electron density and contained some granules with electron-dense cores and clear peripheral zones (Fig. 4A). On day 2, many of these granules showed more prominent clear zones and increased electron density of their central cores; moreover, cytoplasmic disruption had become evident (Fig. 4B). On day 3, there was further disruption of granules, and many cells showed loss of both cytoplasmic and nuclear organization (Fig. 4C). Secondary granules were never observed in any of the cells.

DISCUSSION

The clinical course of this patient with morphologically typical AML was unusual in several respects. She achieved complete remission with relatively little chemotherapy and developed partial remission following an acute bacterial infection. Despite the presence of detectable leukemic cells in her peripheral blood and marked infiltration of blasts in her bone marrow, she remained asymptomatic without significant anemia or thrombocytopenia for
Fig. 4. Electron micrographs of marrow cultures from patient R.G. (A) Cell from relapse culture on day 1. \( \times 16,875 \). (B) Cell from relapse culture on day 2. \( \times 16,875 \). (C) Cell from relapse culture on day 3. \( \times 28,875 \).
nearly a year. Analysis of several series of patients with acute leukemia suggests that remission induction prolongs survival and that this increased longevity is essentially equivalent to the duration of remission. Survival of untreated patients and those refractory to chemotherapy is usually less than 6 mo, and fewer than \(5\%\) will survive 1 yr. Since she received no chemotherapy during this time, a progressive increase in the number of malignant cells and consequent short survival might have been expected. Surprisingly, her peripheral white count remained in the normal range, and her bone marrow was hypocellular for much of this period.

The leukemic cell population in both animal models and humans is a cell renewal system with a tendency to expand. This expansion follows Gompertzian rather than exponential kinetics, implying responsiveness to regulatory mechanisms which decrease growth fraction, and/or increase cell loss as the tumor mass expands. However, death of the host usually occurs before a steady state is achieved. In contrast, the leukemic population in this patient did not seem to be progressively expanding during much of her illness. This unexpected course may have been due to either decreased proliferation or to increased destruction of the malignant cells. The latter seems most likely considering the unique morphological observations in bone marrow cultures. It has been observed consistently that leukemic cells manifest defective maturation in liquid culture systems, and the five other cases of AML reported have behaved as expected in this regard. That is, although overall viability of AML marrow was normal in culture (Fig. 1A), leukemic blast cells persisted without evidence of differentiation or maturation for more than a week in culture (Fig. 2), as also reported by others.

In contrast, the proliferative and maturational patterns in marrow cultures from our unique patient differed strikingly. Morphologically, her bone marrow aspirates were typical of AML. At disease presentation and in relapse, blasts containing Auer rods, vacuoles, and a few azurophilic granules were the predominant cell type. Not more than \(1\%\) of the cells present had characteristics of promyelocytes; therefore it seemed highly unlikely that this case was a variant of acute promyelocytic leukemia. After 1 day in culture, the number of cells identifiable as blasts had dropped by \(86\%\), while the per cent of cells containing granules had increased from \(5\%\) on day 0 to \(81\%\) on day 1 (Fig. 2). The most likely explanation for these morphological changes is that the great majority of the malignant blast cells had differentiated to form granules. However, we cannot exclude the possibility that extraordinarily rapid proliferation of a minor cell population accounted for this marked increase in granulated cells after only 1 day of culture. This formation of large numbers of peroxidase-positive granules by blasts was taken as evidence of cytoplasmic maturation—albeit abnormal maturation since these primary granules were abnormally large and unusually heterogeneous in size, shape, and electron density. Most strikingly, early in culture these granules appeared to lyse spontaneously at a time when viable cell number was dropping precipitously and excessively (Fig. 1A)—a temporal association suggesting that lysosomal disruption may have been toxic to the malignant cells. We believe it reasonable to suggest that the abnormal maturational sequence noted in culture reflects the situation in vivo,
and thereby helps to explain the relatively slow progression of disease in this patient. The findings in vitro in this patient are also relevant to the question of whether or not some patients harbor leukemic cells that can be induced to differentiate normally. The question is of importance, since it has been suggested that the defect in some malignancies is merely a block of normal maturation. If so, therapy should be directed at removing this block, perhaps by some manipulation of cellular environment, rather than at destroying potentially normal cells with highly toxic drugs. In the case reported here, the leukemic cells have shown both impaired and abnormal differentiation. These observations are consistent with the findings of others that in some cases of AML the malignant cells are intrinsically abnormal, rather than normal cells which are impaired by an abnormal environment in vivo. This case also emphasizes, however, that these abnormal cells may retain the ability to differentiate when exposed to an appropriate stimulus.

It would seem important to identify those leukemic patients with cells susceptible to manipulation of their capacity to differentiate, since such patients (as in the reported case) might manifest more indolent courses than usual and therefore require less intensive chemotherapy. Further experience with in vitro studies of leukemic cells may offer the required insights into the extent and nature of the cellular defect in various leukemias, thereby facilitating the design of more rational and individualized therapy in this disease.

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REFERENCES

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