Juvenile Chronic Myelogenous Leukemia: Characterization of the Disease Using Cell Cultures

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To characterize juvenile chronic myelogenous leukemia (JCML), the proliferative properties of bone marrow (BM) and peripheral blood (PB) cells from nine patients were studied using assays for CFU-C and CFU-GEMM and liquid cultures. All specimens showed two reproducible abnormalities: impaired growth of normal hematopoietic progenitors and excessive proliferation of monocytic-macrophage colonies in the absence of exogenous colony-stimulating activity (CSA). Cytogenetic studies in one patient indicated that the CFU-C were malignant because BM cells at diagnosis and monocyte-macrophage colonies showed an abnormal karyotype, whereas PB lymphocytes did not. In contrast to JCML, PB from six adults with Philadelphia (Ph') chromosome-positive chronic myelogenous leukemia (Ph' + CML) yielded CSA-dependent CFU-C colonies which were composed of granulocytes, macrophages, or both, as well as exuberant growth of BFU-E colonies. Co-cultures of JCML BM adherent or nonadherent cells with normal BM resulted in suppression of normal hematopoietic colony formation. Supernatant from JCML adherent cells in liquid culture or plasma from newly diagnosed untreated JCML patients also suppressed control BM colony growth in a dose-dependent manner. These findings confirm that JCML is a malignant disorder of monocytic lineage and suggest that the mechanism of hematopoietic failure in JCML is mediated by an inhibitory monokine secreted by malignant JCML cells.

For control studies, BM was obtained from hematologically normal patients in whom an aspiration was performed as part of a medical investigation or from healthy adult volunteers. PB was also obtained from normal volunteers and from adult patients with classical Ph' + CML. These studies were performed with informed consent and were approved by the Human Experimentation Committee of our institution.

Preparation of cells. Heparinized BM or PB cells were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 g, 4 °C) for 20 minutes to remove neutrophils and RBCs.

Adherent cell fractionation. After Ficoll-Paque, BM mononuclear cells were incubated in plastic Petri dishes or flasks (Falcon Plastics, Becton Dickinson, Oxnard, Calif.) with 10% fetal calf serum (FCS) in a-medium for 90 minutes at 37 °C. In this modification of the technique described by Shaw et al, mononuclear cells were removed by gentle pipetting and repeated vigorous washing of plates with a-medium. The procedure was repeated until no more cells adhered to the Petri dishes. Nonadherent cells harvested in this way contained <3% monocytes as confirmed by the following techniques: microscopic differential count of up to 500 cells prepared with Wright's stain; non-specific (ε-naphthyl butyrate) esterase staining; and immunocytochemical analysis using anti-MY-4 monoclonal antibody (Coulter Immunology, Hialeah, Fla) to identify monocyte-promonocyte cells. In the co-culture studies, normal BM mononuclear nonadherent cells were layered over 1 × 10^6 cells that were adherent to 35-mm Petri dishes.

Liquid culture assay. Adherent PB mononuclear cells from JCML patients, two patients at diagnosis and one patient during remission, were incubated in 25-cm² Falcon flasks with 20% FCS in α-medium at 37 °C with 5% CO₂ in air in a humidified atmosphere. For each study, an appropriate control was performed using PB mononuclear cells (prepared in an identical manner) from five normal donors. After seven days, the supernatants were harvested, frozen at −80 °C, and stored for testing in co-culture studies with control BM.

CFU-C assay. The CFU-C assay was performed as previously described. In brief, nucleated BM cells (10⁶) or PB cells (5 × 10⁶) were cultured in 0.8% methylcellulose, 30% FCS, 20% colony-stimulating activity (CSA), which was prepared from PB leukocytes of a normal volunteer and α-medium. The culture mixture was placed in 35-mm Petri dishes in duplicate and maintained at 37 °C, with 5% CO₂ in air in a humidified atmosphere. Colonies were counted after 14 days using an inverted microscope. A CFU-C colony was defined as a cluster of ≥ 20 cells consisting of granulo-
cytes, monocyte-macrophages, or both. Microscopically, pure granulocytic colonies could be easily distinguished from pure macrophage colonies because of smaller cells and tighter clustering in the former. Individual colonies were plucked from the cultures with a micropipette and spread on glass slides, and the cellular composition was determined microscopically using Wright’s stain and nonspecific (α-naphthyl butyrate) esterase staining with and without treatment with fluoride.4

**CFU-GEMM assay.** The CFU-GEMM assay was performed according to Fauser and Messner.4 In brief, 2 × 10⁶ nucleated BM or PB cells were cultured in methylcellulose with Iscove’s modified Dulbecco’s medium (Ontario Cancer Institute, Toronto), 30% AB serum or plasma obtained from a pool of five normal donors, 5% leukocyte-conditioned medium prepared with phytohemagglutinin (PHA–LCM),26 human erythropoietin 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver) or sheep erythropoietin 2.0 U/mL (Connaught Laboratories, Willowdale, Ontario), and 5 × 10⁵ mol/L of 2-mercaptoethanol. One milliliter of the culture mixture was placed in 35-mm Petri dishes and incubated at 37 °C with 5% CO₂ in air in a humidified atmosphere. All cultures were evaluated after 14 days for the number of BFU-E colonies, defined as an aggregate of >500 hemoglobinized cells or three or more erythroid subcolonies, CFU-C colonies of granulocytic or macrophage cells or both, and mixed colonies containing all elements. Individual colonies were plucked from the cultures with a micropipette and analyzed for cellular composition.

**Cytogenetic analysis.** Cytogenetic studies on fresh BM cells were done by the direct method of Hozier and Lindquist.11 For the study of CFU-C chromosomes, colcemid (0.1 µg/mL) was added for one hour to the culture plates on day 14 of incubation and was then removed by washing with phosphate-buffered saline (PBS). Individual CFU-C colonies were plucked from the culture dishes and pooled, and chromosome analysis was performed by routine methods.11 Banding of chromosomes was done using the trypsin-Giemsa technique.11

**Statistical analysis.** The probability of significant differences between colony numbers was determined by Student’s t test.

## RESULTS

As shown in Table 2, when either PB or BM cells from JCML patients were cultured in the CFU-C assay, the specimens yielded an exceptionally high number of colonies with a plating efficiency of three- to >20-fold higher than that of controls (P < .001). Although colonies from PB and BM from four of seven JCML patients declined when CSA was omitted from the cultures, similar to results with those of controls, the excessive plating efficiency persisted. In three of seven JCML patients, no decline in colony growth was seen when CSA was omitted. Individual JCML colonies at harvest showed all stages of the monocytic lineage, including blast forms, promonocytes, monocytes, and histiocytic macrophages, whereas in controls at least 50% of the colonies were granulocytic. These findings were confirmed by morphological, cytochemical, and immunocytochemical analysis of individual colonies plucked from the cultures and by examination of the cellular composition of entire harvested culture plates.

BM from patient 7 (Table 1) had an abnormal karyotype. In 100% BM cells in metaphase, there were two distal deletions from the long arms of chromosomes 1 and 13 and additional terminal bands on Sq and 12q; the PB lymphocyte karyotype was normal (46 XX). In individual JCML colonies plucked from cultures of PB or BM, the abnormal karyotype was seen in 87% and 100% of the cells in metaphase, respectively. During the patient’s clinical and laboratory remission, cytogenetic studies performed on PB,
BM, and CFU-C revealed a normal karyotype, and CFU-C colony growth was normal in number and dependent on exogenous CSA.

PB colony growth from JCML patients, six adults with Ph\(^{+}\) + CML, and controls is shown in Table 3. All studies were performed on samples depleted of adherent cells and in the presence or absence of added CSA. JCML PB yielded a two- to 14-fold increase in colonies as compared with those of controls (\(P < .0001\)). In sharp contrast to those of controls, JCML colony numbers from five of six patients did not decline when CSA was omitted from the cultures. PB from patients with Ph\(^{+}\) + CML yielded colony numbers higher than those of controls but much lower than those of JCML patients, and the colonies declined 50% when CSA was omitted. Cellular composition of JCML colonies was exclusively monocyte-macrophage, whereas in Ph\(^{+}\) + CML and in controls, it consisted of varying ratios of both granulocytic and monocyte-macrophage cells.

PB from JCML and Ph\(^{+}\) + CML patients was cultured in the CFU-GEMM assay (Table 4). All of the studies were performed on samples depleted of adherent cells and in the presence or absence of PHA-LCM. JCML PB gave rise to excessive growth of monocyte-macrophage colonies that was unchanged when PHA-LCM was omitted from the cultures. No BFU-E or mixed colonies were seen in any cultures of JCML PB whether PHA-LCM was present or not. Ph\(^{+}\) + CML PB also yielded increased CFU-C numbers as compared with those of controls (\(P < .001\)), but the colonies were mainly granulocytic, and they declined ~40% when PHA-LCM was omitted. In comparison to JCML PB, Ph\(^{+}\) + CML PB gave rise to unusually high numbers of granulocytic CFU-C and BFU-E colonies. The BFU-E growth was particularly exuberant and persisted in the absence of PHA-LCM, in contrast to those of controls.

During clinical and laboratory remission, patients 7 and 8 were studied again. PB samples from both yielded normal colony numbers per culture (85 and 58 CFU-C colonies, 50 and 82 BFU-E colonies, 5 and 3 mixed colonies, respectively).

BM from two JCML patients was depleted of adherent cells and co-cultured in varying ratios with control BM that was similarly prepared (Fig 1). With increasing numbers of JCML cells in co-culture, an increase in monocyte-macrophage colony formation was observed, concurrent with a decline of granulocytic CFU-C and BFU-E growth. With co-cultures containing 50% JCML BM cells, BFU-E growth was abolished, and granulocytic CFU-C growth was markedly suppressed.

As seen in Fig 2, adherent JCML BM cells from two patients were left in Petri dishes, and control BM cells were cultured over them in a CFU-GEMM assay. In these cultures, BFU-E, CFU-C, and mixed-colony formation was significantly inhibited as compared with results in studies of control BM cells cultured over control adherent cells (\(P < .01\)).

The supernatant harvested from the liquid cultures of JCML PB adherent cells from two patients was tested for its effect on control marrow colony growth (Fig 3). With increasing concentrations of both JCML supernatants, there was a dose-dependent suppression of CFU-C, BFU-E, and mixed-colony formation as compared with supernatant obtained from flasks of control PB adherent cells that had no effect on cell growth. Supernatant from one of these patients tested during remission yielded results similar to controls.

Figure 4 shows the results of experiments in which various ratios of JCML plasma-control AB plasma were prepared and then substituted in the cultures for control AB plasma that is routinely used in the CFU-GEMM assay. The effect of these preparations was tested on control marrow colony growth. The results from both patients were identical. With increasing percentages of JCML plasma in the cultures, there was a dose-dependent decline in CFU-C and BFU-E colony formation.

### Table 3. Comparison of PB Cell Cultures From JCML and Ph\(^{+}\) + CML Patients Using the CFU-C Assay

<table>
<thead>
<tr>
<th>Patients*</th>
<th>CFU-C/5 x 10^6 Cells Plated</th>
<th>With CSA</th>
<th>Without CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt 1</td>
<td>178 ± 6</td>
<td>192 ± 13</td>
<td></td>
</tr>
<tr>
<td>Pt 2</td>
<td>551 ± 11</td>
<td>380 ± 13</td>
<td></td>
</tr>
<tr>
<td>Pt 3</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td></td>
</tr>
<tr>
<td>Pt 4</td>
<td>156 ± 3</td>
<td>152 ± 4</td>
<td></td>
</tr>
<tr>
<td>Pt 5</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td></td>
</tr>
<tr>
<td>Pt 6</td>
<td>159 ± 2</td>
<td>162 ± 8</td>
<td></td>
</tr>
<tr>
<td>6 Pts with Ph(^{+}) + CML</td>
<td>110 ± 33 (G:M = 3:2)</td>
<td>51 ± 28 (G:M = 3:2)</td>
<td>5 ± 3 (G:M ~1)</td>
</tr>
<tr>
<td>12 Controls</td>
<td>70 ± 29 (G:M ~1)</td>
<td>5 ± 3 (G:M ~1)</td>
<td></td>
</tr>
</tbody>
</table>

*PB, peripheral blood; JCML, juvenile chronic myelogenous leukemia; Ph\(^{+}\) + CML, Philadelphia chromosome positive in addition to CML; PHA-LCM, phytohemagglutinin-leukocyte-conditioned medium; M, macrophage; G, granulocyte.

### Table 4. PB Cultures From JCML and Ph\(^{+}\) + CML Patients in the CFU-GEMM Assay

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>CFU-C</th>
<th>Mixed</th>
<th>BFU-E</th>
<th>CFU-C</th>
<th>Mixed</th>
<th>BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt 7</td>
<td>&gt;1,000</td>
<td>0</td>
<td>0</td>
<td>&gt;1,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pt 8</td>
<td>189 ± 8</td>
<td>0</td>
<td>0</td>
<td>152 ± 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pt 9</td>
<td>137 ± 9</td>
<td>0</td>
<td>0</td>
<td>123 ± 5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 Pts with Ph(^{+}) + CML</td>
<td>146 ± 83 (G:M &gt;1)</td>
<td>8 ± 5</td>
<td>314 ± 153</td>
<td>84 ± 20 (G&gt;M)</td>
<td>0</td>
<td>169 ± 108</td>
</tr>
<tr>
<td>5 Controls</td>
<td>72 ± 16 (G:M =1)</td>
<td>2 ± 1</td>
<td>130 ± 13</td>
<td>37 ± 17 (G:M =1)</td>
<td>0</td>
<td>12 ± 7</td>
</tr>
</tbody>
</table>

*Adherent cell fraction was removed from all specimens prior to plating. The data are presented as mean ± SD.

PB, peripheral blood; JCML, juvenile chronic myelogenous leukemia; Ph\(^{+}\) + CML, Philadelphia chromosome positive in addition to CML; PHA-LCM, phytohemagglutinin-leukocyte-conditioned medium; M, macrophage; G, granulocyte.
Fig 1. Co-cultures of juvenile chronic myelogenous leukemia bone marrow (JCML BM) in varying ratios with control BM in the CFU-GEMM assay. Adherent cells were depleted from all BM specimens prior to culture. Data from two patients with JCML are presented as percentages of control colony numbers. (The mean numbers of control colonies were 125 CFU-C and 130 BFU-E per 10^5 cells plated.) •, Granulocyte CFU-C; ○, BFU-E.

colony numbers. When 90% JCML plasma was substituted for AB plasma, colony growth was almost completely abolished. This inhibitory effect was not detected when remission plasma was tested.

DISCUSSION

There have been only a few research reports on JCML,14-17 and these have been contradictory. Altman et al were the first to claim that juvenile "chronic granulocytic" leukemia is a misnomer, and that the disease is really a panmyelopathy with prominent monocytic involvement and circulating monocyte colony-forming cells. Others disagreed because large numbers of granulocytic CFU-C were demonstrated in cultures of JCML BM and PB.15,16 Our data are clear-cut and resolve this issue. Our major finding in cell cultures of JCML BM and PB was an excessively high number of CFU-C colony numbers in both the CFU-C and the CFU-GEMM culture assay systems. The cellular composition of these colonies was exclusively monocyte-macrophage, as proven by their microscopic appearance in the culture plates, cytospin preparations of harvested plates, morphology, cytochemistry, and surface markers of individual colonies plucked from cultures.

The abnormally high plating efficiency of these monocyte colony-forming cells is most unusual because differential counts of JCML BM and PB prior to culture show granulocytic hyperplasia and only a mild to moderate monocytosis. As shown in Table 1, BM is not "replaced" by monocytic elements as in acute leukemia. Clearly, cells of monocytic lineage in JCML have a unique growth advantage in vitro that is a hallmark of the disease. Because most CFU-C from PB of patients with Ph^+ CML were of granulocytic lineage, confirming other reports,18,19 the in vitro characteristics of JCML were markedly different and emphasize that this condition is a separate entity.

We also found atypical growth requirements for JCML CFU-C. For proliferation of normal BM or PB CFU-C into colonies in vitro, a humoral growth factor is essential and can be provided either by adding exogenous CSA or by leaving intact the adherent cell fraction which produces endogenous
CSA,20 In our studies using CFU-GEMM and CFU-C assays, much of the CFU-C growth from patients with Ph1+ CML and from controls was CSA-dependent, because the colony numbers declined significantly when both adherent cells and CSA were omitted from the cultures. In sharp contrast, added CSA was not essential for JCML CFU-C to proliferate into colonies.

The cytogenetic findings in patient 7 provided good evidence that the JCML monocyte-macrophage CFU-C were involved in the malignant process. The abnormal karyotype was demonstrated in fresh and in cultured BM cells but not in PB lymphocytes. Moreover, when the patient attained a chemotherapy-induced remission, the karyotype could no longer be found, and the BM and PB CFU-C and CFU-GEMM growth characteristics became normal. These results are convincing indications that JCML is a malignant proliferation of monocytic lineage. However, we cannot be certain that the disease is confined exclusively to this lineage.

The CFU-GEMM assay has not been used before to study JCML; we found that it provided new information. Unlike the CFU-C assay, it profiles other hematopoietic lineages and gives more of an overview of cellular events. The CFU-GEMM assay confirmed the high numbers of monocyte-macrophage colony-forming cells. Moreover, JCML CFU-C colonies developed in the absence of an exogenous humoral growth stimulus in this assay, as well as when adherent cells were depleted from the cultured samples. There were also abnormalities of erythropoiesis and mixed-colony formation using standard plating conditions. No detectable BFU-E or mixed colonies were identified in JCML PB, a result clearly different than was found with PB from Ph1+ CML patients and controls. In addition, the hematopoietic growth pattern in vitro became normal during remission, as noted in patients 7 and 8. To assess whether the CFU-GEMM assay has applications as a diagnostic tool or as a monitor of chemotherapy efficacy in JCML, further studies using a wide range of cell concentrations appear to be indicated.

When data shown in Tables 2, 3, and 4 are reviewed, one may ask why normal hematopoietic colony growth did not increase after removal of the inhibitory adherent cell fraction. One explanation is that all pathologic inhibitory cells may not have been adherent. A second explanation is that by depleting the adherent cell layer, we probably removed normal elements that produce CSA and other humoral stimuli, as well as the inhibitory cells. Thus, the net effect was unchanged, and the inhibitory activity prevailed.

In an attempt to explain the growth advantage in vitro of JCML monocyte-macrophage cells and the suppression of BFU-E and granulocytic CFU-C, we studied the interaction of JCML adherent cell fractions and the supernatant from cultures of these fractions. It was clear that the presence of either the adherent cells themselves or the medium in which they were cultured was extremely inhibitory to control BM colony formation, indicating that the JCML adherent cells, which are primarily monocyte-macrophage, produce and secrete a “monokine” that can suppress normal hematopoiesis in vitro. Moreover, by mixing nonadherent BM cells from JCML patients and from controls, we found the same inhibitory effect, suggesting that young undifferentiated JCML cells were capable of excreting the same inhibitory substance. This substance may be the “leukemia inhibitory activity” described by Broxmeyer et al.19,22; however, JCML “monokine” has much more suppressive properties on colony growth. Because plasma from JCML patients also produced inhibition of control BM colony formation, confirming the report of Suda et al.,17 it is likely that the monokine has in vivo relevance and may be an important mechanism of production of the anemia and thrombocytopenia in this disorder.

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