Expression of Embryonic Globins by Erythroid Cells in Juvenile Chronic Myelocytic Leukemia

By Thalia Papayannopoulou, Betty Nakamoto, Nicholas P. Anagnou, David Chui, Lois Dow, and Jean Sanders

Juvenile chronic myelocytic leukemia (JCML) is a rare hematopoietic neoplasia of early childhood with distinct hematologic and biochemical features. We studied the biologic properties and the globin synthetic profiles of JCML erythroid cells both in vivo and in vitro from a total of 24 patients. In these cases we observed the exuberant colony-forming unit-macrophage (CFU-M) colony growth, as reported previously. Furthermore, in contrast to previous reports, we found significant erythroid colony growth in most of our cases (average: 1,182 burst-forming unit-erythroid [BFUe] per 10^3 plated cells, range: 40 to 6,927). This growth was by and large erythropoietin-dependent and was not greatly influenced by other added cytokines. By several criteria all erythroid colony growth detected in vitro was derived from JCML progenitors. The globin synthetic profile of JCML erythroid cells showed high levels of fetal hemoglobin both in vivo and in vitro (fetal: 53% to 94% in reticulocytes, 62% to 98% in BFUe-derived cells). In addition (in seven cases studied) we detected embryonic globins (e and ζ) at the protein and messenger RNA level, a novel finding for primary leukemic cells. We speculate that the transformed erythroid cells in JCML harbor a trans environment supporting expression of developmentally earlier genes (fetal, embryonic). However, in contrast to other acute or subacute leukemias, JCML erythroid cells also have the ability to reach full maturation to the red cell level, thus allowing detection of this primitive program in vivo.

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tor assays the cells were cultured in methylcellulose (MC) in the presence of the following components: 0.9% methyl cellulose (Fisher Scientific, Fairlawn, NJ), 30% fetal calf serum (FCS; Intergen, formerly Armour Pharmaceutical, Purchase, NY), 1% bovine serum albumin (BSA; Calbiochem, San Diego, CA), $10^{-4}$ mol/L 2-mercaptoethanol (Eastman Kodak, Rochester, NY) in Iscove's modified Dulbecco's medium (IMDM; GIBCO Labs, Grand Island, NY). In addition to these culture components, Epo was used at 0.2 to 4 U/mL, interleukin-3 (IL-3) at 3 to 1,500 U/mL, granulocyte-macrophage colony-stimulating factor (GM-CSF) at 10 to 500 U/mL (all from Genetics Institute, Cambridge, MA) and in a few experiments, 10 to 40 U/mL IL-1 (Genzyme, Boston, MA), 50 ng/mL Activin (Genentech, South San Francisco, CA), or 1,000 U/mL of IL-9 were used (Genetics Institute). The number of cells plated varied from 500 to 300,000 per mL.

**Hb Analysis**

**Immunofluorescent studies.** PB smears were subjected to immunofluorescent labeling with previously described23-25 anti-globin chain antibodies (anti-β, anti-γ, anti-ε, and anti-δ). In addition, cytocentrifuge preparations from pooled or single bursts were labeled with the same antibodies.

**Globin chain isoelectric focusing.** For this purpose, fully hemoglobinized bursts were individually plucked from culture plates, pooled, and labeled with 750 μCi/mL §H-leucine (Amersham, Arlington Heights, IL) in the presence of leucine-free media for 12 to 17 hours at 37°C in a 5% CO₂ incubator. Following labeling, they were washed and either used for isoelectric focusing or frozen for later use. Following isoelectric focusing the gels were fixed with En'Hance (DuPont, Boston, MA) and subjected to autoradiography as previously described.26

**Preparation of RNA and S1 nuclease assay.** Total cellular RNA was prepared by cell lysis in 4 mol/L guanidine hydrochloride followed by cesium chloride gradient centrifugation. The RNA pellet was dissolved in 2 mL of NETS buffer (100 mmol/L NaCl, 10 mmol/L Tris hydrochloride [pH 7.5], 1.0 mmol/L EDTA, and 1.0% sodium dodecyl sulfate) and following phenol extraction was precipitated twice in ethanol and stored at −70°C. After centrifugation, RNA was dissolved and used for S1 nuclease analysis. Uniformly labeled M13 MP7 probes specific for β, γ, ε, and ζ globin RNA were used and hybridization conditions under probe excess were as described in detail previously.27

**RESULTS**

**The Hematologic Profile of Patients**

We studied a total of 24 patients, from 1 to 5 years of age, either close to the time of diagnosis or several months after the original diagnosis. All patients were slightly to moderately anemic with a different proportion of total white cells (1,100 to 106,000/mm³) or blasts (4% to 60%) in their blood and most of the patients were thrombocytopenic. Not all tests were performed in all patients. Determination of Hb F in PB was performed in patients not recently (within the last 3 months) transfused (Table 1). Levels of Hb F in those patients ranged from 23% to 51% and their F cells (RBCs containing Hb F) were from 66% to 100% (Fig 1). In some patients repeated determinations were available and showed mild fluctuations over time.

Globin biosynthesis in PB was performed in some of the untransfused patients and showed a fetal (γ) globin level from 53% to 94% with over two-thirds being of the Gy type.

In 11 patients we also had the opportunity to test titers of i and I antigens (by agglutination) and the data are presented in Table 2. As can be seen from these data, the ratio of i to I ranged from 1:4 to 32:1. Thus, i antigen was extremely high and exceeded that of I antigen in several patients, as the ratio was reversed from that normally observed in adults (i:I = 1:64). Almost half of the patients had been treated with prior chemotherapy and the majority had been transfused at some time before our study. With two exceptions (patients 12 and 20), all patients had normal cytogenetics. No patients with monosomy 7 were included.

**Clonogenic Progenitors in JCML and the Effect of Growth Factors**

PB samples were cultured from 16 JCML patients and BM samples from 10 such patients. The number of BFUe per 10⁵ plated cells ranged from 40 to 6,927 (Table 3). The colony growth in four patients was in the upper limit observed in normal individuals (from 40 to 100) but in the rest of the patients it ranged from twofold to 70-fold higher than normal. Patients who had high erythroid colony growth also had exuberant granulocytic/monocytic colony growth, but there were exceptions (Table 3). When a range of plated cells (500 cells to 300,000 cells/plate) was used, it was observed that the erythroid colony growth was usually suppressed in high inocula (eg, 3 × 10⁵), presumably because of overwhelming macrophage growth. Erythroid colonies grown from these patients usually appeared early in culture, ie, during the first week, as unincinctic, pale, translucent colonies with very sharp borders. The degree of hemoglobinization later in culture varied from patient to patient, but there was also variation within a given culture from the same patient.

Endogenous erythroid colony growth, eg, growth in the absence of added Epo, was observed in a minimum of four of nine patients tested (Table 4). Such endogenous erythroid colonies represented (with one exception) a small fraction of the total colonies grown in the presence of Epo.
Fig. 1. PB smear from patient 2 labeled with the anti-\( \gamma \) globin-specific monoclonal antibody. Note the presence of intensely labeled RBCs in a high proportion. Less than 10% of the cells were negative for \( \gamma \) globin.

The effect of added cytokines, eg, IL-3, GM-CSF, IL-6, IL-1, or IL-9, was tested in several cases. The number of BFUe was usually not significantly influenced by IL-3 or GM-CSF (Table 4). Other than removal of monocytes by plastic adherence, there was no further attempt to completely remove other adherent or accessory cells in these cultures.

In one experiment, the replating efficiency of erythroid colonies was tested and the size of the subsequent transferred colonies determined. In this experiment cells from colonies identified as erythroid during the first week were replated in secondary, tertiary, etc. plates. Day 7 colonies ranging in size from 1,500 to 2,000 cells per colony were picked, dispersed, and replated in fresh medium at cell densities from 100 cells per plate to 50,000 cells per plate. Nine days later, or 16 days from day 0, plating efficiencies ranged from 6% to 28%, and the size of the colonies increased. In another set of experiments, intact colonies at day 7 (between 1,500 to 2,000 cells per colony) were plucked and gently transferred without dispersion to plates with fresh media. At day 13 in culture, untransferred colonies left to mature in original plates averaged 6,000 cells in size, whereas transferred colonies averaged 19,000 cells in size. Tertiary transfers from these colonies averaged 29,000 cells in size, whereas similar untransferred colonies were only 22,000 cells in size. Furthermore, the replated colonies were always better hemoglobinized than their stationary counterparts. These replating experiments indicated that proliferative expansion and hemoglobinization in

Table 3. JCML Clonogenic Progenitor Cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sample</th>
<th>BFUe/10(^5)</th>
<th>CFU-GM/10(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB</td>
<td>715</td>
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<td>BM</td>
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<td>3,200</td>
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<tr>
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<td>PB</td>
<td>530</td>
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</tr>
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<td>BM</td>
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<td>280</td>
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<tr>
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<td>BM</td>
<td>40</td>
<td>77</td>
</tr>
<tr>
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<td>PB</td>
<td>3,128* [899]</td>
<td>[488]</td>
</tr>
<tr>
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<td>BM</td>
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<td>840*</td>
</tr>
<tr>
<td>8</td>
<td>PB</td>
<td>2,013</td>
<td>43</td>
</tr>
<tr>
<td>9</td>
<td>PB</td>
<td>1,152, [153], 175*</td>
<td></td>
</tr>
<tr>
<td>10</td>
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<td>360</td>
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<tr>
<td>12</td>
<td>BM</td>
<td>130</td>
<td>90</td>
</tr>
<tr>
<td>13</td>
<td>PB</td>
<td>640 [1,074]</td>
<td>460 [130]</td>
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<tr>
<td>14</td>
<td>BM</td>
<td>570</td>
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<td>PB</td>
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<td>430</td>
</tr>
<tr>
<td>21</td>
<td>PB</td>
<td>610</td>
<td>690</td>
</tr>
</tbody>
</table>

Under optimal culture conditions. Figures in brackets are cultures from frozen cells.

*Normal adult PB.

* Cultures performed 3 months apart.
vitro could improve if colonies were grown away from the influence of background cells.

**Patterns of Globin Synthesis In Vitro**

When globin biosynthesis of BFUe-derived erythroblasts was assessed, the level of Hb F ranged from 62% to 98% and the proportion of ε-γ was from 33% to 77% (Table 5). Although there was variation from patient to patient, values were consistent within the same individual when several bursts were plucked at random and labeled. The globin synthesis of these individual bursts ranged from 79% to 95% Hb F, whereas there was 90% Hb F in a pool of 88 bursts.

We also examined whether the level of Hb F was influenced by fetal sheep serum (FSS), charcoal-treated FCS (FCS-T), or human adult sera, as is the case in BFUe cultures from normal individuals.\(^{28}\) As seen in Table 5, these sera were incapable of modulating Hb F levels in the JCML cultures, in contrast to the data of normal cultures in which FSS or FCS-T virtually abrogate the in vitro activation of Hb F.\(^{30}\) The addition of cytokines (500 U of IL-3, alone or in combination with 300 U IL-6) did not change the pattern of globin biosynthesis (Fig 2).

Pooled bursts from seven patients were also tested by immunofluorescence using antibodies directed against embryonic (ε and ζ), fetal (γ), and adult (δβ) globins (Figs 3 and 4). In all cases studied we were able to detect the presence of a few cells that contained embryonic globins (among pooled burst populations). These cells were not derived by a separate set of bursts because labeling of individual bursts (example shown in Fig 4) showed scattered ε-positive or ζ-positive cells within these bursts. The proportion of ε-positive cells ranged from less than 1% to 13% while the proportion of ζ-positive cells ranged from about 2% to 30% (Table 6). Presence of embryonic globins was confirmed at the RNA level using S1 nuclease mapping (Fig 5). In one case, fresh blasts were used for S1 nuclease assay and ε messenger RNA (mRNA) was detected (Fig 5).

**DISCUSSION**

One of the hallmarks of JCML is the abundant endogenous clonal growth of predominantly myelo-monocytic progenitors in vitro along with the prevalence of immature monocytoid cells in vivo.\(^4,7\) This "endogenous" or "spontaneous" growth of monocytic colonies in vitro appears to be dependent on the presence of adherent cells or their products and, therefore, is considered to be nonautonomous.\(^{31}\) Whether the specific overproduction of IL-1 or GM-CSF are the factors responsible, as previously suggested, is unclear.\(^{2,23}\) Enhanced sensitivity to GM-CSF and/or to an as yet unidentified growth factor with quantitative or qualitative aberrations in the expression of the growth factor receptor could also be the underlying mechanism.\(^{3,34}\) The heightened sensitivity to Epo, which we observed here, may also be considered as part of the altered pattern of responsiveness of JCML progenitors. Further, endogenous colony-forming unit-macrophage (CFU-M) growth, as previously described, was present in the cases
more, in our replating experiments we noted that both the growth and hemoglobinization of erythroid bursts improved. The exuberant erythroid growth observed in our patients was largely Epo-dependent, although a usually small proportion of colonies could be grown in cultures without exogenously added Epo. Such “endogenous” or Epo-“independent” colonies have been also found in monosomy 7, in other erythroleukemias, and in chronic myeloproliferative disorders. Such results have been studied here. However, this type of in vitro growth has been observed in other myelomonocytic leukemias of early childhood, including monosomy 7 or myelodysplasias, that may or may not be variants of JCML.

In previous JCML studies in which the exuberant monocytic/macrophage colonies were noted, erythroid growth has been described infrequently and only in isolated cases. Furthermore, failure to grow erythroid colonies has been emphasized as one of the distinguishing features of the disease, including inhibition of normal erythroid progenitors by JCML cells. In contrast to these data, we have uncovered significant (several-fold higher than normal) erythroid colony growth in the majority of our cases. The reasons for differences between the previously reported data and ours are not readily apparent. We suspect that differences in culture components and inocula do play a role. High inocula or overwhelming growth of JCML CFU-M progenitors suppress erythroid growth.

Fig 2. Isoelectric focusing of globin chains from lysed 3H-leucine-labeled bursts. Control samples are from cord blood (CB) and K562 cells to indicate mobility of adult, fetal, and embryonic chains. Lanes numbered 1 to 5 are pooled bursts from patient 2 cultured in the presence of FCS, except for number 3, in which the FCS was replaced by FSS. All five samples contained Epo. Additionally, lane 1 had IL-3 and IL-6, lanes 2 and 3 had IL-3, and lane 4 had IL-6. The preponderance of fetal globin synthesis is evident in all of these samples. In addition, all samples show visible ζ bands, whereas ε is detectable only in some of them.

Fig 3. Cytocentrifuge smears of pooled BFUe-derived cells from patient 2. Note the high intensity of labeling with anti-γ (A) and the presence of positive cells with anti-ε (B) and anti-ζ (C) antibody.
interpreted to indicate hypersensitivity to Epo. Although maturation levels varied from patient to patient the endogenous erythroid colonies were usually poorly hemoglobinized, as is the experience with the other disease states (eg, PV, CML). It is also possible that their numbers are underestimated because of our inability to detect them (ie, lack of hemoglobinization).

The globin synthetic profile of BFUe-derived JCML colonies was the main focus of our study. The majority of the cases studied showed over 80% Hb F by globin biosynthesis. Such high levels of γ globin have not been observed before in cultures of adult individuals and, in fact, are even higher than those usually measured in newborn progenitors. It is of interest that in almost all of the cases we studied the proportion of γ chains was over 50%. Thus, these globin synthetic profiles are reminiscent of the ones seen in late gestation fetal livers, or in premature neonates.

In addition to fetal globin, noted in isolated reports before, we detected the expression of the embryonic globins, ε and ζ, at the protein and mRNA level. This finding has not been previously reported for primary leukemia cells. However, embryonic globins are abundantly produced in erythroleukemic lines established from the cells of patients with leukemias. As shown in Fig 5, ε RNA was present both in primary noncultured leukemia cells (lane 1) from one patient and in BFUe-derived erythroblasts from five patient samples. The expression of ζ globin was slightly more abundant (Table 6) than the expression of ε in immunofluorescence assays of pooled JCML bursts, but in no case was there only one of the two embryonic chains present. It is of interest that ε and ζ were not synthesized exclusively by a distinct set of progenitors, because cells expressing them were scattered among other fetal producing cells in several single bursts studied. All cases that produced some embryonic globin had high levels of Hb F in vivo. Likewise, other fetal characteristics, ie, increased i, decreased I, and decreased carbonic anhydrase, were found in cases with high levels of Hb F in their RBCs.

The presence of both ε and ζ embryonic globins in the JCML erythroblasts is intriguing and suggests that JCML cells, in addition to their unique growth characteristics, possess a “trans” environment conducive not only to high levels of fetal globin and/or other fetal characteristics (i, CA-1) but also of embryonic globin expression. It is of interest that we failed to detect embryonic globin in the mature RBCs of these patients in vivo (data not shown), possibly because of subthreshold levels in these terminal cells. Recently, ε or ζ mRNA has been found by polymerase chain reaction (PCR) in adult samples; however, no embryonic proteins were detected in these studies.

By several criteria the abundant erythroid colony growth observed in our cases is believed to be derived from leukemic rather than from normal erythroid progenitors: (1) their morphology (tight, single rather than multi-clustered colonies) and their poor tendency to hemoglobinize were distinct from normal; (2) the level of fetal globin was extremely high, which reflected the high levels of Hb F found in the PB of these patients; (3) a significant number of “endogenous” colonies can be present in many of these patients; and (4) as is the case with fetal cells, FSS or FCS-T failed to influence the levels of Hb F synthesized by these colonies. Normal progenitor growth, if present in these cases, remained undetected.

One of the interesting questions is why high Hb F is found with some consistency in JCML. Initially it was suggested that this phenomenon reflected the transformation of a rare cell found postnatally that has escaped the full developmental switch (from γ to β) during the perinatal period and that is responsible for the production of Hb F-containing cells (F cells) in adult life. However, several pieces of evidence gathered subsequently suggested that the fetal to adult developmental changes in erythroid cells are mediated not through changes in cell lineages but in programs within a single lineage, and that Hb F production is an inherent, facultative property of adult stem cells. Also, transformation of incompletely “switched” cells in utero does not appear likely because cases diagnosed soon after birth as opposed to the cases diagnosed a few years later do not appear to have higher levels of Hb F (Table 1). Although the nature of the transformation event or the target cells involved remains obscure, we speculate that what may distinguish JCML from the other acute or subacute leukemias in which

![Fig 4. A flattened portion of a burst showing scattered positive cells for ζ globin.](image-url)
erythroid cells participate is not only that high levels of Hb F are produced by the leukemic cells, but the fact that their maturation is not compromised. The reasons for this successful in vivo maturation in JCML are unclear at present. It is possible that transformed cells in JCML produce or respond to factors in addition to EpO that enhance erythroid maturation. Further studies in this intriguing entity may provide insights about the biologic behavior of JCML erythroid cells.

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