Chronic Myelocytic Leukemia (CML): Failure to Detect Residual Normal Committed Stem Cells In Vitro

By Jack W. Singer, Philip J. Fialkow, Laura Steinmann, Vesna Najfeld, Sidney J. Stein, and William A. Robinson

Granulocytic colonies grown in culture from marrow and peripheral blood from five patients with Ph' positive CML and heterozygous at the G-6-PD locus were analyzed for G-6-PD in order to identify CFU-C that do not arise from the CML clone. The patients had both B and A enzymes in normal tissues, but their CML clones typed as B. Whereas about 50% of colonies from normal subjects heterozygous as the G-6-PD locus show type-A G-6-PD and 50% type B, only two of the 1308 colonies from the CML patients had type-A G-6-PD. These data provide little evidence for persistence of normal committed stem cells in CML, a finding in contrast to that made previously in polycythemia vera, another clonal stem cell myeloproliferative disorder.

CHRONIC MYELOCYTIC LEUKEMIA (CML) is a neoplastic disorder characterized predominantly by an excessive overgrowth of granulocytic cells. Although clinically the megakaryocytic and the erythroid series are only variably involved, studies using glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes and other markers (reviewed in ref. 4) indicate that the erythrocytes, granulocytes, platelets, and macrophages all arise from the CML pluripotent stem cell clone. Utilization of the G-6-PD marker for this purpose is based upon the fact that in accordance with inactivation of one X chromosome in each somatic cell, females heterozygous at the X-linked G-6-PD locus for a B and an A gene have two populations of cells—one synthesizing type-A and the other type-B enzyme. In a G-6-PD heterozygote, if a malignant disease arises from a single cell, the progeny of that cell will have either type-A or type-B activity, but not both.

Each of 12 G-6-PD heterozygotes with CML studied in our laboratory had both B and A enzymes in normal tissues but only a single enzyme type in CML cells, indicating that the disease had clonal origin at the time of investigation (4 patients typed as A and 8 as B). The CML myelocytic cells from the 5 patients who are the subjects of this report were type B.

The object of the present work was to use G-6-PD as a marker to determine whether or not there are residual normal committed stem cells (CFU-C) in patients with CML, as was demonstrated recently for the related disorder polycythemia.
To this end, G-6-PD was analyzed in large numbers of individual granulocytic colonies grown from blood and marrow using several sources of colony-stimulating factor (CSF). Elsewhere we reported studies that indicate such colonies grown from a normal G-6-PD heterozygote show either B or A G-6-PD, strongly suggesting a unicellular origin for each colony. Presumably, in the G-6-PD heterozygotes with CML who had type-B enzyme in their leukemia clones, granulocytic colonies derived from residual normal CFU-C would show type-A as well as type-B G-6-PD.

There is some evidence suggesting persistence of some normal stem cells in CML. Approximately 20% of patients treated with aggressive chemotherapy convert, at least transiently, to Ph' negativity. The assumption underlying the suggestion that there are residual normal stem cells based upon the appearance of Ph'-negative cells in these studies is that a stem cell that lacks Ph' is normal. Studies of granulocytic colonies with G-6-PD have two advantages over earlier studies using Ph' as a marker: (1) it is conceivable that leukemogenesis is a multistep process and that Ph' occurs in an already clonal marrow; (2) larger numbers of colonies can be studied.

MATERIALS AND METHODS

Study subjects. Five patients with Ph'-positive CML were studied. Clinical and blood cell G-6-PD data on patients 3 and 4 were reported in detail previously. Peripheral blood and relevant clinical data on the five subjects with CML at the time of CFU-C culture are shown in Table 1. In each of the patients, all CML hematopoietic cells typed as B, but cultured skin fibroblasts showed both enzyme types.

Peripheral blood from CML patients 1 and 2 was drawn in preservative-free heparin in Atlanta and Denver, respectively, and shipped by air to Seattle; elapsed time between blood drawing and culturing was 8–12 hr. Specimens from patients 3 and 4 were obtained in Johannesburg and shipped by air to Seattle at room temperature; the elapsed time between aspiration and culture was 36–72 hr.

Spleen cell suspensions from patient 5 were prepared within 60 min of surgical removal. The spleen was placed in Ca2+- and Mg2+-free Dulbecco phosphate-buffered saline (PBS) (Gibco) and diced into small fragments with a scalpel. Single-cell suspensions were made by serial passage through an 18- and then a 22-gauge needle. Samples were washed twice in PBS and transported in RPMI-1640 with 20% inactivated fetal calf serum (FCS). Separation was performed by density gradients (Teva, Jerusalem), and the cells were cultured as described for the other samples.

Our CFU-C culture techniques have been reported elsewhere. For these studies, sources of CSF included serum from patients undergoing acute graft-versus-host disease, PHA-conditioned medium, and feeder layers of peripheral blood leukocytes prepared by the method described by Pike and

<table>
<thead>
<tr>
<th>Patient/Age (yr)</th>
<th>Duration of Therapy (mo)</th>
<th>Cells/mm³</th>
<th>Myeloblasts (%)</th>
<th>Promyelocytes (%)</th>
<th>Hematocrit (%)</th>
<th>Ph' (Cells, No. Studied/No. Positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/32</td>
<td>12</td>
<td>59,900</td>
<td>1</td>
<td>7</td>
<td>39</td>
<td>20/20</td>
</tr>
<tr>
<td>2/30</td>
<td>8</td>
<td>27,400</td>
<td>0</td>
<td>3</td>
<td>40</td>
<td>12/12</td>
</tr>
<tr>
<td>3/63</td>
<td>30</td>
<td>54,300</td>
<td>0</td>
<td>0</td>
<td>36</td>
<td>30/30</td>
</tr>
<tr>
<td>4/31</td>
<td>30</td>
<td>6,990</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>28/28</td>
</tr>
<tr>
<td>33</td>
<td>33</td>
<td>13,600</td>
<td>0</td>
<td>0</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>36</td>
<td>33,500</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>5/38</td>
<td>22</td>
<td>18,600</td>
<td>1</td>
<td>5</td>
<td>28</td>
<td>801/80</td>
</tr>
</tbody>
</table>

*Direct preparation of marrow or blood. Ph' was studied at the time of diagnosis in patients 3 and 4 and at the time of patient study in the other patients.

† Sum of direct preparation of blood (30 cells) and spleen (50 cells).
Marrow or peripheral blood mononuclear cells were cultured (2 × 10⁶/ml) in 0.8% methylcellulose (MCB), 20% FCS (Reheis), and alpha medium (Flow Laboratories) containing penicillin and streptomycin. The cell-containing medium was either overlaid on a 0.5% agar (Bactoagar, Difco) feeder layer of peripheral blood cells or plated with 0.1 ml of a source of CSF in 10 × 35 mm Petri dishes (Lux) and incubated for 10-14 days at 37°C in a high-humidity 5% CO₂–95% air tissue culture incubator. For each patient studied, at least 20 random colonies were picked from the same plates used for G-6-PD studies and identified as granulocytic by Wright-Giemsa staining and peroxidase positivity.

To perform the electrophoretic analysis of G-6-PD, individual granulocytic colonies were harvested under direct vision with a dissecting microscope. Intact colonies were harvested randomly, and care was taken to avoid closely adjacent colonies, which might have arisen from two spatially paired progenitors. Each colony was lifted from the methylcellulose medium into a glass capillary tube and placed in a drop of saline containing 0.006 M NADP on a glass slide. The bulk of the medium was teased away from the colony with gentle washing. Individual colonies were then picked up again in a micropipette and placed on cellulose acetate strips presoaked in electrophoretic buffer. Three colonies were placed onto each strip—two from experimental cultures and one from cultures grown under identical conditions from a hematologically normal white control with type-B G-6-PD. Lysis of the colonies was achieved by freezing on dry ice. The strips were immediately subjected to electrophoresis and then stained for G-6-PD activity. At least 50 colonies in each experimental group were harvested on each patient.

RESULTS

The electrophoretic results on the colonies from the five CML patients are shown in Table 2. Over 95% of the colonies could be scored for G-6-PD, and each colony was characterized by a single isoenzyme. CSF source had no effect on the G-6-PD type of subsequently harvested colonies. Only 2 of 1308 colonies were type A.

DISCUSSION

The single-enzyme phenotypes of CFU-C from normal heterozygotes provides direct confirmation of the previously assumed but only indirectly proven hypothesis that human granulocytic colonies are unicellular in origin. If colonies originated from more than one cell, variable quantities of both enzyme types would be present. Only progeny of a single cell would consistently type as either A or B.

As in preliminary studies of only a few colonies, our current data provide scant evidence for persistence of normal stem cells in patients with CML. The patients were studied both in early and late chronic phase as well as in poor and good control. In patients 1-4, enough colonies were electrophoresed to determine that at least 99% of CFU-C were from the CML clone.

It is highly improbable that the 36–72-hr delay prior to culture of the samples from South Africa resulted in preferential loss of non-CML clonal CFU-C.

Table 2. Granulocytic Colonies From CML Patients: G-6-PD Isoenzyme Analysis

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of Type-B Colonies</th>
<th>No. of Type-A Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>309</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>126</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>256</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>570</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1306</td>
<td>2</td>
</tr>
</tbody>
</table>
Patients 1 and 2, whose cells were cultured within 8 hr, and the spleen from patient 5, which was cultured within 1 hr, had no non-CML clonal CFU-C, whereas 2 of 828 colonies from patients 3 and 4 were not of the CML clone.

The findings in CML contrast with the observations in polycythemia vera, another myeloproliferative disorder involving clonal proliferation of pluripotent marrow stem cells. In two patients with polycythemia vera, granulocytic colonies not derived from the abnormal clone and therefore presumably normal were detected in the blood in a ratio of 30% for one patient and slightly under 5% for the other.5 In contrast, in the five G-6-PD heterozygotes with CML reported here whose CML clones typed as B, only 2 of 1308 colonies were found with type-A G-6-PD, giving a ratio of 0.15% normal colonies, a highly significantly different result from the ratios in the polycythemia vera patients ($\chi^2 \approx 15, p < 0.001$).

One possible explanation for the observed differences in frequency of normal stem cells between CML and polycythemia vera is a difference in the rates of disease progression. The earliest that we studied granulocytic colonies in CML was 8 mo after diagnosis, and we did not detect normal stem cells. On the other hand, normal CFU-C were found in two polycythemia vera patients first studied 4 and 8 yr after diagnosis and then restudied at 6 and 10 yr. However, the frequency of non-clonal CFU-C dropped significantly in the 2-yr interval.19 If the rate of disease progression in CML is more rapid than in polycythemia vera, it is possible that only studies on newly diagnosed patients can detect a nonclonal CFU-C population.

Ph1 has also been used as a marker to study CFU-C. The presence of some normal stem cells in even advanced CML was suggested by the observation of a few Ph1-negative granulocytic colonies in three of five patients.8 However, other investigators have found such colonies to be uniformly Ph1 positive.9 11 If Ph1-negative CFU-C do persist in CML, this fact must be reconciled with our finding of only CML clonal-type CFU-C. Two hypotheses must be considered: (1) only some patients have Ph1-negative CFU-C (e.g., the patients studied by Chervenick et al.8 but not those studied in this and other three reports9 11); (2) there may be some Ph1-negative CFU-C in all patients. If the latter hypothesis is correct, then the G-6-PD data suggest that cells clonally derived acquire Ph1 at a later stage in leukemogenesis or that it occurs secondarily after leukemic transformation. This hypothesis was suggested by the possibility that some lymphocytes derived from the CML clone were Ph1 negative.3 20 This question can be resolved by studies of CFU-C using both Ph1 and G-6-PD as markers simultaneously.

Our data suggest that the primary defect in CML is establishment of an abnormal stem cell clone with a growth advantage over normal stem cells. In contrast to observations in polycythemia vera, we found little evidence for persistence of normal stem cells in CML. We do not know if such stem cells are absent or if they are present but suppressed in a manner such that they are not readily detectable in vitro. In any event, the results do demonstrate a fundamental difference between polycythemia vera and CML.

ACKNOWLEDGMENT

We thank Dr. Robert Jacobson, Dr. Johan Kriel, Dr. Mark Lowenthal, Dr. Clive Sinoff, and Peta Basford for their help in obtaining specimens from Johannesburg. This work would not have been possible without the expert technical assistance of M. C. James and C. Ernst.
REFERENCES

Chronic myelocytic leukemia (CML): failure to detect residual normal committed stem cells in vitro

JW Singer, PJ Fialkow, L Steinmann, V Najfeld, SJ Stein and WA Robinson