Selective Expansion of Primitive Normal Hematopoietic Cells in Cytokine-Supplemented Cultures of Purified Cells From Patients With Chronic Myeloid Leukemia

By A.L. Petzer, C.J. Eaves, M.J. Barnett, and A.C. Eaves

We have previously reported that primitive normal hematopoietic cells detectable as long-term culture-initiating cells (Ph-LTC-IC) are present at high levels in the blood of some patients with chronic myeloid leukemia (CML). We now show that this population can be expanded several-fold when highly purified CD34+CD38- cells isolated from the blood of such patients are cultured for 10 days in a serum-free medium containing 100 ng/mL of Flt3-ligand and Steel factor and 20 ng/mL of interleukin-3 (IL-3) and IL-6, and granulocyte colony-stimulating factor. In similar cultures initiated with CD34+CD38- cells from CML blood samples in which all of the LTC-IC were leukemic (Ph+), Ph+ LTC-IC activity was rapidly lost both in the presence and absence of admixed CD34+CD38- cells isolated from normal marrow. Conversely, the ability of normal LTC-IC to expand their numbers was shown to be independent of the presence of Ph+ LTC-IC and later types of Ph+ colony-forming cell (CFC) progenitors. In contrast to the LTC-IC, CFC were consistently amplified in cultures initiated with CML-derived CD34+CD38- cells and the additional CFC present after 10 days were, like the starting population of CFC, almost exclusively Ph- regardless of the genotype(s) of the LTC-IC in the original CML samples. Amplification of the Ph+ CFC population in these cultures showed the same factor dependence as previously demonstrated for the in vitro expansion of CFC from normal marrow CD34+CD38- cells. Ph+ LTC-IC disappeared regardless of the cytokines present. Taken together these findings support a model of CML in which the leukemic stem cells are characterized by a decreased probability of self-renewal and an increased probability of differentiation. In addition, they suggest new opportunities for improving the treatment of CML using strategies that require autologous stem cell rescue.

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CHRONIC MYELOID leukemia (CML) is a hematologic malignancy that is believed to arise in a hematopoietic stem cell with lymphoid and myeloid differentiation potential.1 The disease is characterized biologically by the deregulated overgrowth of the marrow by a multilineage clone of cells, all of which are marked by the presence of a novel BCR-ABL fusion gene.2 The formation of this new gene usually involves a reciprocal translocation between the long arms of chromosomes 9 and 22 leading to the formation of a unique chromosome identified as the Philadelphia chromosome (Ph).3 The transcription of the BCR-ABL gene leads to the synthesis of a new protein that has been shown to have transforming properties in a variety of model systems.1,9 In patients, a marked expansion of the leukemic (Ph+) clone is accompanied by an excessive production of mature granulocytes and a concomitant suppression of normal hematopoiesis. Nevertheless, a residual population of primitive normal (Ph-) cells persists in many patients.10-13 These cells may be found in the blood in patients studied soon after diagnosis or during the period of endogenous hematopoietic recovery that follows myelo-reductive therapy14 and include cells with functional properties associated with stem cells; ie, cells with transplantable hematopoietic regenerative potential15 and that are able to produce in vitro colony-forming cells (CFC) for at least 5 weeks in cocultures containing adherent stromal cell feeder layers.10,14 The latter are referred to as long-term culture-initiating cells (LTC-IC).15 In addition to Ph+ LTC-IC, variable numbers of Ph+ LTC-IC are also found in the marrow and blood of CML patients.13,14 However, at all later stages of myeloid differentiation Ph- cells typically predominate.14,18

Normal and leukemic LTC-IC are not readily distinguished with respect to their expression of most surface markers thus far examined. These include CD34, CD71, CD38, and Thy-1, whose upregulation or downregulation among both types of LTC-IC exists.25,26 Thus, isolation of various subfractions of the CD34+ cells present in CML blood samples (eg, the CD34+CD71+CD38- subpopulation) can provide a source of cells that is highly enriched in LTC-IC, but their predominant genotype remains the same as in the original unfractionated blood sample and this can be either Ph+ or Ph-.14 Even where differences in the relative proportion of leukemic and normal LTC-IC in a given subpopulation have been reported (eg, in the case of CD34+HLA-DR- cells14,25), phenotypic heterogeneity among both types of LTC-IC exists.25,26 Thus, isolation of a particular phenotype may not provide the resolving power necessary to obtain clinically useful CML autografts. An alternative purging approach under current clinical evaluation is based on the rapid loss of Ph+ LTC-IC that is seen when CML blood or marrow cells are cocultured on stroma under conditions that maintain (but do not expand) normal LTC-IC.29 Because we recently found that LTC-IC from normal marrow can be significantly expanded in cultures containing soluble cytokines,23,30 it was of interest to determine whether the Ph+ LTC-IC often present in isolates of...
Highly purified CD34+ CD38- cells (>99% pure) were isolated by FACS from selected cryopreserved samples of light density (<1.077 g/mL) blood cells of 4 different CML patients because they had been previously shown to contain elevated numbers of exclusively Ph' LTC-IC and CFC. The cells were incubated for 10 days at 37°C in a serum-free medium containing FL and SF at 100 ng/mL each, and IL-3, IL-6, and G-CSF at 20 ng/mL each. At the end of 10 days, LTC-IC and CFC assays were performed on the harvested cells.

RESULTS

Rapid loss of Ph' LTC-IC activity with expansion of Ph' CFC in serum-free cultures of CD34+ CD38- CML cells. Table 1 shows the results of a first series of experiments to investigate how the number of Ph' LTC-IC (and CFC) might change in cultures of highly purified CD34+ CD38- CML cells maintained for 10 days under serum-free conditions in the presence 100 ng/mL of Flt3-ligand (FL) and SF, plus 20 ng/mL of IL-3, IL-6, and G-CSF. This combination of cytokines was used because it had been previously found to stimulate a large expansion of both LTC-IC (≈25-fold) and CFC (≈800-fold) in analogous cultures of CD34+ CD38- cells isolated from normal marrow.30 As shown in Table 1, these conditions supported a rapid expansion of the CFC population in cultures initiated with exclusively Ph' CD34+ CD38- cells (although the extent of this expansion was highly variable). Interestingly, in the same 10-day-old CML cultures, Ph' LTC-IC activity was consistently lost (no detectable LTC-IC activity in cultures from three patients, ie, a >1,000-fold decrease, with an ≈50-fold reduction measured in the fourth case).

Because we had previously found that different cytokine combinations are required to maximize the in vitro expansion of CFC and LTC-IC from normal CD34+ CD38- marrow cells,30 the possibility that the various component cytokines, either alone or in other combinations, might have had a different effect on Ph' CD34+ CD38- cells was investigated. However, as shown in Table 2, the same rapid loss of Ph' LTC-IC activity seen with the five-cytokine cocktail (Table 1) occurred regardless of the cytokine(s) added. On the other hand, the results for Ph' CFC expansion (Table 3) paralleled those previously obtained for the expansion of CFC in cultures of normal CD34+ CD38- marrow cells36; ie, IL-3 was the most effective single cytokine that could stimulate an amplification of the Ph' CFC population, and the combination of FL plus SF and IL-3, although more effective than any one of these three alone, was not as effective as when IL-6 and G-CSF were also present (compare results in Table 3 with those shown in Table 1).
whether an expansion of the normal (Ph⁺) LTC-IC seen in some CML blood samples could be obtained under conditions that had been found to cause a rapid loss in detectable Ph⁺ LTC-IC (Table 1). To investigate this question, we used a different set of five cryopreserved CML blood cell samples in which the LTC-IC had been previously found to be predominantly Ph⁺ (only Ph⁺ LTC-IC detected), although the coexisting CFC were exclusively Ph⁻. The numbers and genotypes of the LTC-IC and CFC obtained from 10-day suspension cultures initiated with the CD34⁺CD38⁻ cells isolated from these samples are shown in Tables 4 and 5, respectively. All of these cultures contained the same five-cytokine cocktail used in the experiments described in Table 1 (100 ng/mL of FL and SF, and 20 ng/mL of IL-3, IL-6, and G-CSF). In two cases (nos. 6 and 7), the Ph LTC-IC had increased fourfold to fivefold by the end of the 10-day culture period. In the third case (no. 5), a fivefold increase in Ph LTC-IC was obtained in a first experiment but not in a repeat experiment in which the initial yield of LTC-IC was considerably lower. In the fourth case (no. 8), the Ph LTC-IC declined slightly and, in the fifth case (no. 9), where again the LTC-IC content of the starting population of CD34⁺CD38⁻ cells was unusually low, their numbers were not sustained. CFC numbers were expanded in all cultures and, unexpectedly, in every case, were predominantly Ph⁻ (Table 5). The fact that this occurred in the absence of detectable Ph⁻ LTC-IC indicates that the new CFC present in such 10-day cultures were derived either from CD34⁺CD38⁻ cells initially detectable as CFC and/or from some otherwise uncharacterized CD34⁺CD38⁻ cell type that is not detectable as either a CFC or as an LTC-IC. The fact that very few Ph CFC were produced in cultures that contained exclusively Ph⁻ LTC-IC suggests that the majority of the expanded populations of CFC obtained within the first 10 days in cultures of normal CD34⁺CD38⁻ cells also represent the progeny of cells that are not initially detectable as LTC-IC. However, in neither instance do these findings exclude the possibility that many (or even most) normal or Ph⁻ LTC-IC can give rise to small numbers of CFC within 10 days under these culture conditions because such cells would represent a population too small to be detected by the level of discrimination possible by cytogenetic analysis of individual colonies.

To determine whether the different responses exhibited by Ph⁺ and Ph⁻ progenitors in cultures of CD34⁺CD38⁻ CML blood cells might be influenced by potential interactions between Ph⁺ and Ph⁻ cells, a series of mixing experiments was performed. In each of these, cultures were initiated with Ph⁺ CD34⁺CD38⁻ cells isolated from different CML blood samples and then mixed with an equal number of CD34⁺CD38⁻ cells obtained from a sample of marrow from a normal individual. For these experiments, additional aliquots of cells from the first two patients studied in Table 1 (nos. 1 and 2) and the first patient studied in Table 4 (no. 5) were used. The results obtained (Table 6) show no evidence of a negative effect of Ph⁺ cells on the ability of normal LTC-IC to be amplified in suspension cultures containing the same five-cytokine cocktail (FL, SF, IL-3, IL-6, and G-CSF) used in the described experiments previously. Conversely, the presence of CD34⁺CD38⁻ cells from normal marrow had no salvaging effect on the concomitant rapid loss in vitro of Ph⁺ LTC-IC activity.

### DISCUSSION
The present experiments show that previously cryopreserved, normal (Ph⁻) LTC-IC isolated from the peripheral blood of some CML patients at diagnosis could be expanded several-fold when incubated for 10 days under highly defined culture conditions (Table 4), although the expansion

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#### Table 4. Increase in Normal (Ph⁻) LTC-IC in 10-Day Cultures of CD34⁺CD38⁻ CML Blood Cells

<table>
<thead>
<tr>
<th>CML No.</th>
<th>Day 0</th>
<th>% Ph⁻</th>
<th>Day 10</th>
<th>% Ph⁻</th>
<th>Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>18</td>
<td>100</td>
<td>2 (2/2)³</td>
<td>93</td>
<td>100 (11/11)</td>
</tr>
<tr>
<td>5b½</td>
<td>100</td>
<td>200</td>
<td>4 (4/4)</td>
<td>&lt;0.03</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>102</td>
<td>100</td>
<td>22/22</td>
<td>467</td>
<td>100 (26/26)</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>100</td>
<td>17/17</td>
<td>47</td>
<td>100 (25/25)</td>
</tr>
<tr>
<td>8</td>
<td>196</td>
<td>100</td>
<td>20/20</td>
<td>87</td>
<td>100 (1/1)</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>100</td>
<td>4/4</td>
<td>&lt;0.0004</td>
<td>—</td>
</tr>
</tbody>
</table>

Cells were incubated in a serum-free medium containing FL and SF at 100 ng/mL each, and IL-3, IL-6, and G-CSF at 20 ng/mL each.

* All LTC-IC numbers in this study were determined by dividing the total CFC detected in the 6-week-old LTC-IC assay cultures by 8 based on preliminary data suggesting that this represents the average CFC output per Ph⁻ LTC-IC cultured under the conditions used here (unpublished data, 1996). The corresponding average CFC output from normal cytokine-mobilized LTC-IC is 25.³¹ Therefore, the values shown in this table represent overestimates of absolute Ph⁻ LTC-IC frequencies by up to threefold. However, this would be unlikely to affect the expansion values shown because we have previously shown that the CFC output per LTC-IC does not change when normal LTC-IC are expanded in cultures containing the cytokines used here.²³

† Values shown in parentheses are the numbers of LTC-IC-derived colonies scored as Ph⁻ divided by the total number of LTC-IC-derived colonies analyzed.

‡ For explanation of a and b experiments, see footnote to Table 4.

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#### Table 5. Production of Ph⁺ CFC From CD34⁺CD38⁻ CML Blood Cells in Which All Initial LTC-IC Were Ph⁺

<table>
<thead>
<tr>
<th>CFC (per 100 input cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML No.</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>5a</td>
</tr>
<tr>
<td>5b½</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
</tbody>
</table>

Same experiments as in Table 4.

* Values shown in parentheses are the numbers of colonies scored as Ph⁺, divided by the total number of colonies analyzed.

† For explanation of a and b experiments, see footnote to Table 4.
Table 6. The Presence of Ph+CD34+CD38+ CML Cells Has No Effect on the Expansion of Normal LTC-IC in 10-Day Cocultures

<table>
<thead>
<tr>
<th>Source of CML Cells Added</th>
<th>No. of Day 10 LTC-IC per 100 Input CML Cells Cultured in the Presence of 100 Normal BM Cells</th>
<th>% Ph+ Day 10 LTC-IC</th>
<th>Expansion of Normal LTC-IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML no. 1 (M)</td>
<td>&lt;0.025</td>
<td>100 (21/21, F)</td>
<td>10 x</td>
</tr>
<tr>
<td>CML no. 2 (F)</td>
<td>&lt;0.025</td>
<td>100 (27/27, F)</td>
<td>42 x</td>
</tr>
<tr>
<td>CML no. 5 (M)</td>
<td>&lt;0.025</td>
<td>97 (33/34, F)†</td>
<td>45 x</td>
</tr>
<tr>
<td>None</td>
<td>450</td>
<td>—</td>
<td>17 x</td>
</tr>
</tbody>
</table>

All cultures contained CD34+CD38+ cells isolated from a normal female (F) bone marrow (BM) sample and 100 ng/mL of FL and SF, and 20 ng/mL of IL-3, IL-6, and G-CSF. CD34+CD38+ CML cells were added as shown.

Abbreviation: ND, not done.

* Day 10 LTC-IC value relative to number of LTC-IC in 100 input normal BM cells (±27).
† In the cocultures of normal female marrow plus CML no. 3 cells, 1 Ph+ (male) colony was found in the LTC-IC assays.

was less than what we have seen in analogous cultures of CD34+CD38+ cells isolated from the marrow of normal individuals. Further studies will be required to determine whether a reduced proliferative potential may be a generalized feature of circulating or mobilized LTC-IC relative to the normal marrow LTC-IC population or whether this is a unique feature of circulating Ph+ LTC-IC in CML patients. The variable yields of expanded Ph+ LTC-IC in culture suggest that there may be considerable heterogeneity in the numbers of functionally different subtypes of LTC-IC (or their precursors) that are spontaneously mobilized in different CML patients. Under the same culture conditions, Ph+ LTC-IC were unable to maintain their functional state. This defective behavior of Ph+ LTC-IC could be seen in cultures containing a variety of cytokine supplements (Tables 1 and 3) and occurred independent of whether or not normal LTC-IC were present (Table 6). In previous studies, we have shown that Ph+ LTC-IC decrease 50-fold more rapidly than do normal LTC-IC, both under conditions used to initiate long-term cultures from bone marrow cells and when the cells are seeded onto preestablished marrow adherent layers. Under either of these conditions, which do not involve the provision of exogenous soluble cytokines, normal LTC-IC are maintained at input levels for 7 to 10 days, but do not expand. The fact that a marked loss of LTC-IC activity can also be demonstrated when Ph+ LTC-IC are cultured in highly purified form and at low cell densities suggests that this abnormal behavior is intrinsically determined and not readily modulated by extracellular factors, including those involved in the interactions such cells have with stroma.

The mechanism underlying the loss of LTC-IC activity exhibited by cultured Ph+ cells has not yet been determined. For example, whether the loss of this activity is associated with cell death or, alternatively, with a change in proliferative potential, acquisition of an ability to proliferate in methylcellulose and/or cytokine responsiveness are all questions that will need to be addressed in future studies. Some groups have obtained evidence to indicate that the BCR-ABL gene product can confer an increased resistance to the apoptotic response that is normally induced by factor deprivation and others have suggested that CML cells may be characterized by a tendency to differentiate prematurely. We have recently shown that circulating Ph+ LTC-IC represent a rapidly cycling population, in contrast to the LTC-IC that circulate in normal individuals, or the Ph- LTC-IC that are sometimes also detectable in the blood of CML patients. In addition, we have recently shown that BCR-ABL mRNA is present at detectable levels in all types of primitive Ph+ cells regardless of their phenotype. Taken together, these findings support a model of CML in which the Ph+ stem cell has two linked features: (1) an intrinsically increased ability to remain alive and proliferate under conditions that may promote the apoptosis or quiescence of normal stem cells, and (2) a decreased probability of self-renewal, ie, an increased probability of differentiation. Such a model could account for a number of observations of the behavior of the leukemic clone in vivo. These include the relatively long average latent period (≈7 years) before Ph+ clones typically attain a size sufficient for the development of clinical symptoms of disease, the delayed relapses (after >5 years) that are occasionally seen in CML patients previously “cured” of their disease by an allogeneic bone marrow transplant, and the rapid but transient resurgence of predominantly Ph+ hematopoiesis in patients given combination chemotherapy without a transplant.

Finally, the present experiments draw attention to the possibility of using cell separation in combination with defined culture conditions to improve current culture-purging approaches to reducing the leukemic stem cell burden in CML autotransplants. A procedure that involves a significant reduction in the number of cells to be cultured and, hence, the volumes to be handled could greatly facilitate the development of more widely applicable, safer, and cheaper closed systems for the routine manipulation of such transplants. The added advantage of being able to simultaneously amplify the normal stem cell content of the graft while purging residual CML stem cells would also be particularly attractive for use in patients where the starting population of normal stem cells may be limiting, or where a gene transfer procedure is envisaged.

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