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# Rapid decline of chronic myeloid leukemic cells in long-term culture due to a defect at the leukemic stem cell level

(hematopoietic stem cells/self-renewal/hematopoiesis)

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**ABSTRACT** **In this report we describe a quantitative *in vitro* assay for the most primitive type of leukemic precursors yet defined in patients with chronic myeloid leukemia (CML). This assay is based on the recently described "long-term culture-initiating cell" (LTC-IC) assay for primitive normal human hematopoietic cells. Such cells, when cocultured with competent fibroblast feeder layers, give rise after a minimum of 5 weeks to multiple single and multilineage clonogenic progenitors detectable in secondary semisolid assay cultures. Similar cultures initiated by seeding a highly enriched source of leukemic cells from patients onto normal feeders showed the clonogenic cell output after 5 weeks to be linearly related to the input innoculum over a wide range down to limiting numbers of input cells, thus allowing absolute frequencies of leukemic LTC-ICs to be determined using standard limiting dilution analysis techniques. Leukemic LTC-IC concentrations in CML marrow were found to be decreased, on average to <10% of the normal LTC-IC concentration in normal marrow, but were greatly increased (up to >$10^5$ times) in CML blood. Assessment of the number of clonogenic cells produced per leukemic LTC-IC by comparison to normal blood or marrow LTC-IC values showed this function to be unchanged in leukemic LTC-ICs [i.e., 3.1 ± 0.4 clonogenic cells per CML LTC-IC (mean ± SEM, $n = 6$) versus 3.7 ± 1.2 ($n = 3$) and 4.3 ± 0.4 ($n = 5$), respectively, for normal blood and marrow LTC-ICs]. In contrast, leukemic LTC-IC maintenance in LTC proved to be highly defective by comparison to normal LTC-IC of either blood or marrow origin. Thus, when cells from primary LTC were subcultured into secondary LTC-IC assays, leukemic LTC-IC rapidly declined (>30-fold) within the first 10 days of culture, whereas normal LTC-IC numbers remained unchanged during this period. These findings illustrate how self-maintenance and differentiation events in primitive human hematopoietic cells can be differentially modulated by an oncogenic process and provide a framework for further studies of their manipulation, analysis, and therapeutic exploitation.**

Chronic myeloid leukemia (CML) is a multilineage clonal hematopoietic malignancy characterized by excessive production of granulocytes and the presence in the leukemic cells of a consistent rearrangement of the *BCR* (breakpoint cluster region) and *ABL* [Abelson murine leukemia viral (v-*abl*) oncogene homolog] genes, typically manifested in metaphase preparations as the Philadelphia chromosome ($Ph^1$) (1). The initial cell transformed, and hence the origin of the leukemic clone, is believed to be a totipotent hematopoietic cell with lymphoid as well as myeloid differentiation potential since $Ph^1$-positive cells in these lineages are frequently demonstrable (2). This has suggested that production of the BCR–ABL kinase in a totipotent hematopoietic cell gives it a selective growth advantage. Recent experiments involving retroviral infection of murine bone marrow (BM) cells with BCR–ABL constructs are consistent with this (3, 4), although an underlying molecular mechanism has not been determined. In particular, the biological consequences of BCR–ABL kinase expression in very primitive human hematopoietic cells have been difficult to investigate because methods for their selective isolation have not been available.

CML patients with elevated leukocyte (WBC) counts show dramatic increases in the number of $Ph^1$-positive clonogenic progenitors in their circulation (5, 6) and we have shown that continued production of $Ph^1$-positive clonogenic cells for many weeks can occur at a high level when peripheral blood cells from such patients are cultured on irradiated marrow cell adherent layers established from normal individuals (7). Recently, we showed that the number of clonogenic cells present after 5–8 weeks in similar cultures initiated with *normal* hematopoietic cells allows the detection of a very primitive class of clonogenic cell precursors that exhibit properties characteristic of cells with long-term *in vivo* reconstituting potential (8–10). These normal human "long-term culture-initiating cells" (LTC-ICs) can be quantitated by limiting dilution analysis, which then allows the proliferative potential of individual LTC-ICs to also be determined (11). We now show that this approach can be applied to detect and quantitate leukemic LTC-ICs from patients with CML and that these cells exhibit similarities and differences in their behavior by comparison to normal LTC-ICs.

## MATERIALS AND METHODS

**Cells.** Heparinized BM aspirate cells were obtained with informed consent from normal individuals and $Ph^1$-positive CML patients undergoing marrow harvests for transplantation. Heparinized blood from additional normal individuals and from CML patients undergoing routine hematologic assessment were similarly obtained. All CML patients were $Ph^1$-positive and in stable chronic phase. For the initial experiments with CML blood, only samples containing >20 × $10^9$ WBCs per liter were used, as this allows selection of patients whose circulating $Ph^1$-positive progenitors are sufficiently elevated that even after maintenance in LTC only $Ph^1$-positive cells are detected (7) (see also Fig. 3*A*), thus avoiding the necessity for confirmatory progenitor genotyping as is required for similar experiments with CML BM (12). BM cells for initiation of LTC were used either without further processing or after removal of contaminating erythrocytes when the nucleated cell count in the original speci-

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Abbreviations: LTC-IC, long-term culture-initiating cell; CML, chronic myeloid leukemia; WBC, leukocyte; BM, bone marrow; $Ph^1$, Philadelphia chromosome; BFU-E, burst-forming unit, erythroid; CFU-GM, colony-forming unit, granulocyte/macrophage; CFU-GEMM, CFU, granulocyte/erythroid/macrophage/megakaryocyte.

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men was $<2 \times 10^7$ cells per ml. For clonogenic cell assays, erythrocytes were first lysed by brief exposure to ammonium chloride (13), and the cells then were washed twice in Iscove's medium with 2% fetal calf serum. For blood cell samples, light-density ($<1.077$ g/cm$^3$) cells were isolated by centrifugation on Ficoll/Hypaque either with (normal blood) or without (CML blood) T-cell depletion. This involved incubation of the light-density cells with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes for 30 min on ice followed by further centrifugation at 4°C on Ficoll/Hypaque to remove the rosetted T cells (14). These were reduced, on average, by this procedure by >98% according to FACScan analysis of CD2-positive cells in a lymphocyte-gated population. The primary purpose of T-cell removal was to prevent the spontaneous activation and outgrowth *in vitro* of Epstein virus-transformed B lymphocytes, which preliminary experiments showed occurs regularly within 5 weeks when inadequately T-cell-depleted normal peripheral blood samples are cocultured with irradiated marrow adherent layers. Since the T-cell content of the high WBC count peripheral blood specimens obtained from CML patients was already decreased to a few percent, further removal of T cells from these samples was not undertaken and development of spontaneous transformants was not encountered.

**LTCs.** Test cells were cultured in LTC medium (15) at varying initial cell concentrations in dishes or wells containing (or not containing) a standardized number of irradiated (15 Gy) normal marrow adherent layer cells subcultured from primary LTC (8) according to the particular experimental design. All LTCs were maintained at 37°C for the first 3–4 days and then subsequently at 33°C. After the first 7–10 days half of the medium and half of the nonadherent cells were removed and replaced with new LTC medium. This feeding procedure was then repeated after 14 days and at weekly intervals thereafter (15). For all LTC-IC assays, cultures were initiated on normal marrow feeder layers (as described above) and then maintained for 5 weeks. At this time all of the nonadherent cells and the trypsinized adherent cells were harvested, washed, and plated in methylcellulose cultures for quantitation of clonogenic cells (see below). For LTC-IC maintenance studies, the entire contents of primary LTC were harvested at the times indicated, aliquots were used to initiate secondary LTC on new irradiated feeders, and these secondary LTCs were then maintained a further 5 weeks prior to harvesting and plating of the cells in methylcellulose assays. The number of clonogenic cells detected at this time provided a relative measure of the LTC-ICs in the primary LTC at the time they were harvested. This value was then normalized by the number of LTC-ICs detected in primary LTC-IC assays of the original cell suspension, to yield a percent input value.

**Colony Assays.** Erythropoietic (burst-forming unit, erythroid; BFU-E), granulopoietic (colony-forming unit, granulocyte/macrophage; CFU-GM), and multilineage (CFU, granulocyte/erythroid/macrophage/megakaryocyte; CFU-GEMM) progenitors were assayed by plating test cell suspensions at appropriate concentrations in replicate methylcellulose cultures under standardized conditions optimized for expression of the colony-forming potential of these cells as assessed after 18–21 days of incubation at 37°C using previously described colony-scoring criteria (16). In all assays of fresh or cultured CML marrow, colonies produced in methylcellulose were genotyped by cytogenetic analysis of individually removed single or small pools of colonies (17) to allow the proportion of normal and leukemic clonogenic cells (and hence LTC-ICs) to be determined.

## RESULTS

**Assays for CML LTC-ICs.** We have previously defined LTC-ICs as cells that give rise to clonogenic hematopoietic cells (i.e., BFU-E, CFU-GM, and/or CFU-GEMM) detectable after a period of initial culture for 5 weeks on competent feeder layers (11). Such feeders can be provided in a standardized form by subculturing into the LTC-IC assay cultures a fixed number of cells per unit area using 2- to 6-week-old normal marrow-derived LTC adherent layer cells as a source of feeder cells. The validity of using the clonogenic cell-producing property of LTC-ICs as an endpoint for their quantitation depends, however, on the existence of a linear relationship between the number of LTC-ICs seeded into the cultures and the number of clonogenic cells present 5 weeks later, regardless of the input LTC-IC concentration. This was previously demonstrated for assays of LTC-ICs in normal marrow cell suspensions (11) and in T-depleted light-density cell suspensions from normal blood (18).

Our previous studies had shown that LTC initiated with peripheral blood cells from CML patients with high WBC counts [and a marked elevation in circulating Ph$^1$-positive progenitors (5)] when analyzed 4–8 weeks later contained high numbers of exclusively Ph$^1$-positive clonogenic progenitors (7) in contrast to LTC initiated with CML BM (12). This suggested that it might be possible to use CML peripheral blood from such patients as an enriched source of primitive leukemic cells to investigate the relationship between cell input and leukemic clonogenic cell output 5 weeks later. In a series of eight such experiments (with samples from eight different patients with high WBC counts) in which the number of light-density peripheral blood input cells was varied from $5 \times 10^3$ to a maximum of $10^7$ cells per 2.5 ml LTC (in 35-mm tissue culture dishes), the slope of the line relating the logarithm of the innoculum size (total nucleated cells) to the logarithm of the number of clonogenic cells detected after these LTCs had been maintained for 5 weeks was $1.05 \pm 0.21$ (which is not significantly different from 1.0; mean ± SEM, $P > 0.5$). The results for a representative patient are illustrated in Fig. 1. Thus, conditions developed for normal LTC-IC appear to also be suitable for the detection and quantitation of Ph$^1$-positive LTC-IC.

**Measurements of Leukemic LTC-ICs in CML Blood and Marrow.** Because the relative output of clonogenic cells from circulating leukemic LTC-ICs was found to be constant under the assay conditions used, even down to limiting numbers of input cells (e.g., see Fig. 1), quantitation of absolute leukemic LTC-IC numbers by limiting dilution analysis was possible. For such experiments, blood from CML patients with elevated WBC counts was again used as a highly and selectively enriched source of leukemic LTC-ICs. Irradiated normal marrow-derived feeders were subcultured into 96-well flat bottom Nunclon plates (11) and then from 50 to $2 \times 10^5$ light-density cells were added per well in volumes of 100 μl with 23 ± 1 wells per group. Five weeks later, all of the cells in each well were suspended and plated in methylcellulose assay cultures to enable detection of one or more clonogenic cells per well. From the proportion of positive and negative LTCs defined in this way, the frequency of LTC-ICs in six different input samples was calculated using Poisson statistics (19, 20). Results for a representative experiment are shown in Fig. 2. From this, the average 5-week output of clonogenic cells per Ph$^1$-positive LTC-IC was then derived in each case. The results of this latter calculation are shown in Table 1 together with results obtained when the same procedure and assay conditions were used to analyze LTC-ICs in normal marrow (11) or blood (18). The proliferative potential of all of these types of LTC-IC, as assessed by this 5-week clonogenic cell output endpoint, can be seen to be similar and relatively constant, providing further support for the use of the LTC-IC assay to quantitate and characterize a very primitive Ph$^1$-positive cell type.

Knowledge of the 5-week clonogenic cell output per leukemic LTC-IC allows absolute values to be derived from total

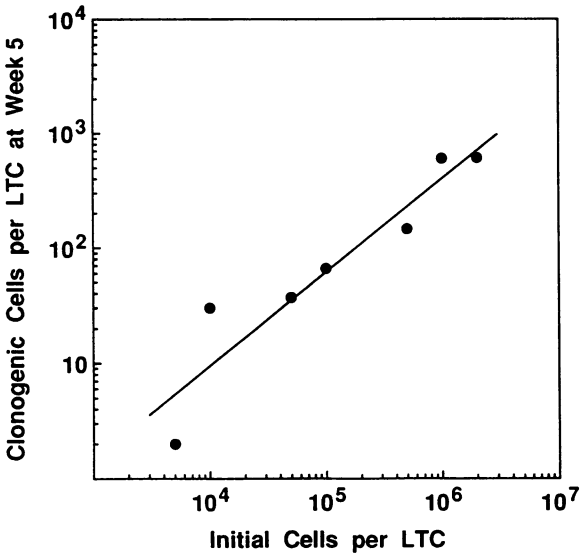


FIG. 1. Linear relationship between the number of light-density CML peripheral blood cells seeded into individual LTCs (containing irradiated preestablished normal marrow feeders) and the number of clonogenic cells detected in secondary methylcellulose assays of cells harvested from these primary LTCs 5 weeks after their initiation. Each point represents a single LTC. All points are derived from a single representative experiment using cells from a CML patient with a WBC count of 190 × $10^9$ per liter. The slope of the regression line fitted to this data set is 0.81 ± 0.13.

5-week clonogenic cell yields measured in cultures initiated with nonlimiting innocula, which are experimentally easier to perform than limiting dilution analyses. LTC-IC values were thus obtained for peripheral blood samples from an additional 20 CML patients, and the concentration of LTC-ICs per ml of blood was then calculated assuming 100% LTC-IC recovery in the light-density fraction assayed (8). The results are shown in Fig. 3 together with circulating LTC-IC values

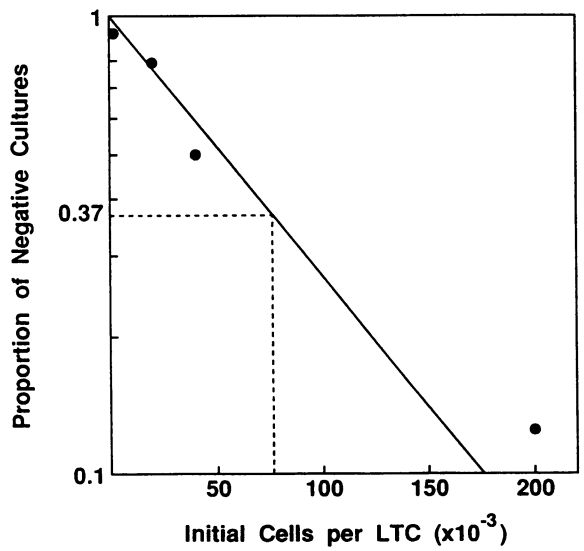


FIG. 2. Limiting dilution analysis of data from a representative experiment in which decreasing numbers of light-density CML peripheral blood cells (from a patient with a WBC count of 21 × $10^9$ per liter) were seeded onto irradiated marrow feeders and the cultures were then assayed 5 weeks later for the presence (positive cultures) or absence (negative cultures) of ≥1 clonogenic cell. In this experiment, the frequency of LTC-ICs in the suspension assayed (i.e., the reciprocal of the concentration of cells that gave 37% negative cultures) was 1 per 7.6 × $10^4$ cells (95% confidence limits = 1 per 5.3 × $10^4$–1 per 11.0 × $10^4$).

Table 1. Proliferative potential of normal and leukemic LTC-ICs

| Sample | No. of clonogenic cells per LTC-IC after 5 weeks | No. of experiments |
|---|---|---|
| Normal BM | 4.3 ± 0.4 | 5 |
| Normal blood | 3.7 ± 1.2 | 3 |
| CML blood | 3.1 ± 0.4 | 6 |

Values are expressed as mean ± SEM from *n* experiments. The frequency of LTC-ICs in each experiment (determined by limiting dilution assays) was multiplied by the total number of cells plated to determine the total number of LTC-ICs assayed in that experiment. The total number of clonogenic progenitors after 5 weeks was similarly derived from clonogenic progenitor assay data. The number of clonogenic cells per LTC-IC was then calculated by dividing the total number of clonogenic cells present after 5 weeks by the total number of LTC-ICs assayed.

obtained from similar measurements of T-depleted, light-density peripheral blood samples from a large series of normal individuals (18). In Fig. 3*A*, LTC-IC concentrations in CML blood are plotted as a function of the WBC count. It can be seen that LTC-IC numbers increase exponentially such that values >$10^5$-fold higher than normal circulating LTC-IC levels are seen in patients with the largest tumor burdens. In Fig. 3*B*, the number of circulating LTC-ICs in individual CML patients is plotted as a function of the number of circulating clonogenic cells (BFU-E plus CFU-GM plus CFU-GEMM per ml) in the same patient. On average, leukemic LTC-ICs were found to circulate at a 10-fold lower frequency than clonogenic cells, although these two parameters showed a highly significant association (Spearman's rank correlation coefficient, $r_s = 0.77$; $P < 0.0001$; $n = 26$). By comparison, the ratio of circulating LTC-ICs to clonogenic cells in normal blood appears much lower (≈1:80).

LTC-IC assays were also performed using CML marrow samples. However, in each of these experiments, cytogenetic analyses were performed on the colonies produced from the clonogenic progenitors present after 5 weeks in LTC to distinguish $Ph^1$-positive and $Ph^1$-negative LTC-ICs as, in contrast to CML blood, $Ph^1$-positive LTC-ICs would be anticipated to frequently represent a minority population relative to normal LTC-ICs in CML marrow (12, 21). As predicted by previous studies, the concentration of $Ph^1$-positive LTC-ICs (relative to other nucleated cells) in the 12 CML marrows analyzed was quite variable and in general markedly reduced, by comparison to LTC-IC values in control marrows (i.e., ≤2.8 ± 1.4 $Ph^1$-positive LTC-ICs per $10^6$ CML marrow cells as compared to 55 ± 12 LTC-ICs per $10^6$ marrow cells from normal individuals, $n = 13$) and by comparison to normal ($Ph^1$-negative) LTC-ICs coexisting in the same CML marrows tested (for which a value of 5.4 ± 1.2 per $10^6$ cells was obtained).

**Differential Maintenance of Normal and Leukemic LTC-ICs in Culture.** In previous studies we have found that normal marrow LTC-ICs are well maintained in LTC established from a single input innoculum (22, 23) and similar kinetics are seen when highly purified LTC-ICs from normal marrow are seeded onto preestablished feeders (24). Fig. 4 shows the corresponding results obtained when light-density peripheral blood cells from CML patients with high WBC counts were seeded onto irradiated human marrow feeders and the number of LTC-ICs was then followed by harvesting these primary LTCs and performing secondary LTC-IC assays (as described in *Materials and Methods*). For comparison, analogous experiments were performed for primary LTC established by seeding light-density, T-cell-depleted normal peripheral blood or normal marrow buffy coat cells onto preestablished marrow feeders. It can be seen that normal LTC-IC maintenance in such cultures was the same regard-

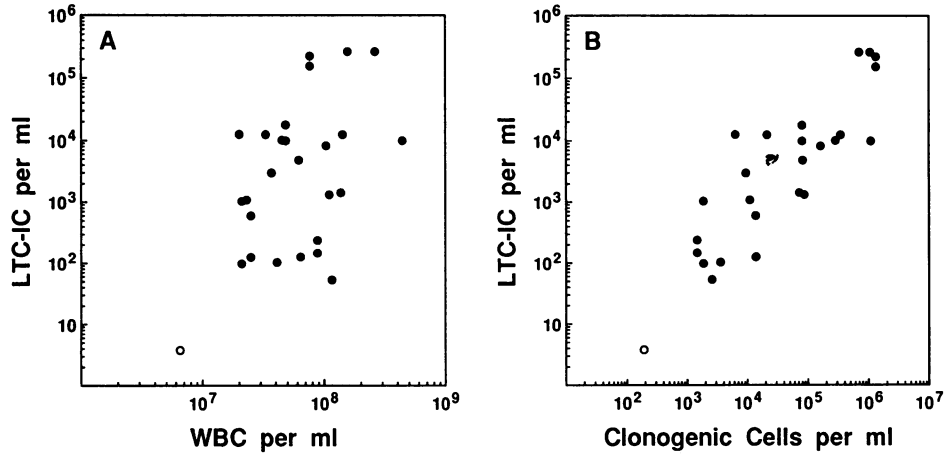


FIG. 3. LTC-IC concentration (per ml) in the peripheral blood of different CML patients (solid circles) as compared to 23 normal individuals (the open circle in each panel shows the mean ± SEM of 2.9 ± 0.5 LTC-ICs per ml measured in these individuals) as a function of the WBC count (per ml) (*A*) or the peripheral blood clonogenic progenitor (BFU-E plus CFU-GM plus CFU-GEMM) content per ml (*B*). Absolute LTC-IC values were obtained either directly by limiting dilution analysis or indirectly from the total clonogenic cell output measured at week 5 divided by the average number of clonogenic cells produced per LTC-IC—i.e., 3 and 4 for CML and normal LTC-ICs respectively, as described in the text. A significant association between the two parameters measured in *B* is indicated by a Spearman's rank correlation coefficient, $r_s = 0.77$ ($P < 0.0001$).

less of the source of LTC-ICs with no decrease in overall population size during the first 10 days. In contrast, the leukemic LTC-IC population showed an immediate and rapid rate of decline down to ≈3% of input values within the same initial period during which time the cultures had not been manipulated in any way except to reduce the temperature from 37°C to 33°C.

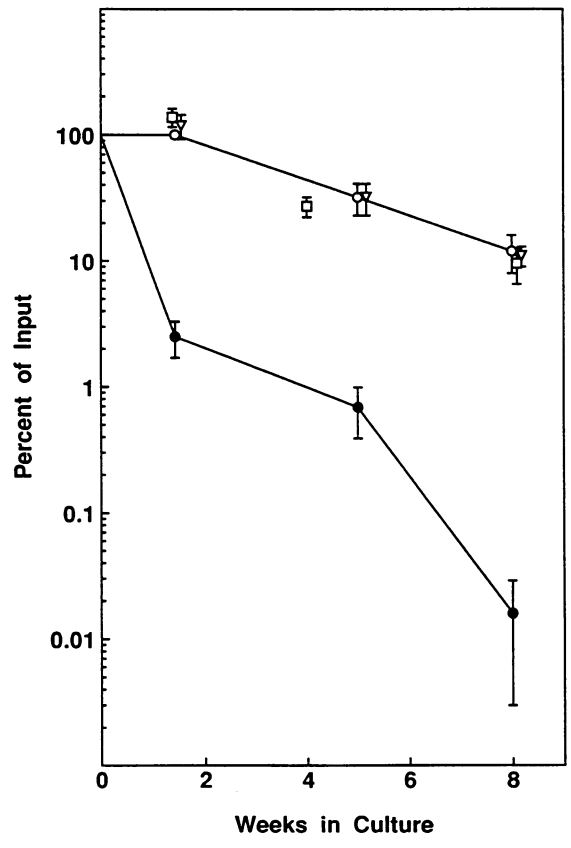


FIG. 4. Differential kinetics of CML (solid symbols) versus normal (open symbols) LTC-ICs in LTC initiated from cells seeded onto irradiated normal marrow feeders. Values shown are mean ± SEM after normalization of data in individual experiments by setting LTC-IC values in the primary innoculum in each experiment to 100%; $n = 6$ for CML (peripheral blood LTC-ICs), $n = 5$ for LTC-ICs in normal blood (open circles), and $n = 2$ for LTC-ICs in normal marrow (open triangles). Open squares show previously published data for LTC-ICs in normal unseparated marrow cultured in the absence of preestablished feeders (23).

## DISCUSSION

In this report we describe the development and initial use of a quantitative assay for a primitive Ph[1]-positive cell that meets the definition of a LTC-IC—i.e., a cell that after a minimum period of 5 weeks in culture, together with certain marrow adherent cells but in the absence of exogenous growth factors, will have produced detectable clonogenic progenitors. Appropriate purification studies cannot yet rule out the possibility that some LTC-ICs (either normal or leukemic) are also detectable in either standard direct clonogenic assays or in the blast colony assays described by Leary and Ogawa (25) or Gordon *et al.* (26). However, it should be noted that none of these has used such a prolonged interval prior to assessment of clonogenic cell production and, indeed, if this requirement were imposed, would decrease considerably the quoted frequencies of any of these clonogenic cell types. Additional operational advantages of the LTC-IC assay are its relative simplicity, ease of standardization, and applicability to quantitation of primitive hematopoietic cells in primary patient samples. The need for selective identification of particular colony subtypes in primary or secondary assays is avoided and the requirement to use subcultured irradiated normal human marrow feeder layers can be met by substituting murine fibroblasts (24).

By restricting our initial studies to examination of peripheral blood samples from CML patients with elevated WBC counts, the problem of contaminating normal LTC-ICs contributing to the results was circumvented as this proved to be a source of highly enriched Ph[1]-positive LTC-ICs (7) (Fig. 3). Ph[1]-positive LTC-ICs were found to produce, on average, a similar number of clonogenic cell progeny after 5 weeks in LTC as do their normal counterparts in the blood or marrow of normal individuals. However, a number of abnormalities in the CML LTC-IC population was also revealed. First, their distribution between marrow and blood was shown to be grossly altered, even more dramatically than is the case for Ph[1]-positive clonogenic cells. Both populations increase exponentially in the blood with linear increases in the WBC

count, but $Ph^1$-positive LTC-IC appear to be present at relatively reduced frequencies in CML marrow whereas $Ph^1$-positive clonogenic cell frequencies in CML marrow are relatively normal (27). Second, in spite of a normal output of clonogenic cell progeny by $Ph^1$-positive LTC-ICs and the provision of a preestablished feeder derived from a normal marrow donor, their initial maintenance in the LTC system was highly compromised relative to normal LTC-IC. Whether this is due to an intrinsic defect in the $Ph^1$-positive LTC-IC that is not subject to extrinsic modulation and/or whether such differences may also prevail *in vivo* have yet to be determined. However, it is interesting to speculate that the behavior of normal and leukemic LTC-ICs in the LTC system may indicate how these cells behave *in vivo* under analogous conditions of stimulation. One might then expect to see evidence of a growth advantage of the stem cells in the $Ph^1$-positive clone *in vivo* only when most coexisting normal stem cells were in a quiescent state. The latter might be anticipated to occur in chronic phase CML patients managed with conventional therapy, but a situation more closely resembling that obtained in LTC might occur *in vivo*, albeit transiently, following more intensive treatment. It is interesting to note that clinical experience fits well with these predictions (28, 29).

In human LTC, primitive normal clonogenic cells in the adherent layer alternate weekly between a quiescent and a dividing state (16), and in murine LTC, it has been possible to demonstrate that extensive proliferation of some long-term totipotent reconstituting cells does occur (30). In LTC initiated with $Ph^1$-positive LTC-ICs, their derivative primitive clonogenic progeny divide continuously, suggesting a defective, unregulated mechanism for inevitable expansion of the $Ph^1$-positive clone. Thus the LTC system may serve as an important model for further dissection of the mechanisms that regulate normal versus CML recovery patterns *in vivo*.

The present studies also suggest a simple explanation for the previous, apparently paradoxical finding that $Ph^1$-positive cells often rapidly decline in LTCs initiated with CML marrow (12) but not with CML blood (7). It is now clear that the ratio of leukemic to normal LTC-IC numbers in these two sources of hematopoietic cells may differ over many orders of magnitude. Thus a decline of $Ph^1$-positive clonogenic cells to undetectable levels within the first 5 weeks in a LTC initiated with a CML marrow would be expected if the frequency of leukemic LTC-ICs were very low (i.e., less than one in the number of cells used to initiate each culture). Nevertheless, it is interesting to note that $Ph^1$-positive LTC-ICs are also selectively disadvantaged in the LTC system, providing a strong rationale for the continued use of this approach to purge autologous CML marrow autografts (21, 31). Preliminary studies in our laboratory have also shown that most $Ph^1$-positive LTC-ICs have features expected of activated cells (32), in contrast to normal LTC-ICs which show features of quiescent cells (8, 9). The availability of a quantitative assay for a very primitive $Ph^1$-positive cell population should thus make possible a variety of studies to further characterize these cells, to obtain a better estimate of the number of leukemic stem cells in individual CML patients, and to devise more effective treatment strategies *in vivo* and *ex vivo*.

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