Characterization of Primitive Hematopoietic Cells in Normal Human Peripheral Blood


The total number of clonogenic cells present in 5-week-old long-term cultures (LTC) initiated by seeding normal human marrow cells on competent adherent cell feeder layers allows for the quantitation of a more primitive hematopoietic input precursor cell type referred to as an LTC-initiating cell (LTC-IC). Previous studies have suggested that LTC-IC also circulate because production of clonogenic cells continues for many weeks when cells from the light-density (<1.077 g/mL), T-cell-depleted fraction of normal blood are maintained on irradiated, marrow-derived feeder layers in LTC medium. We now show that the number of clonogenic cells present in such reconstructed LTC after 5 weeks is linearly related to the input number of peripheral blood (PB) cells over a wide range of cell concentrations, thereby permitting the quantitation of circulating LTC-IC by limiting dilution analysis. Using this approach, we have found the concentration of LTC-IC in the circulation of normal adults to be 2.9 ± 0.5/mL. This is approximately 75-fold lower than the concentration of circulating clonogenic cells (ie, burst-forming units-erythroid plus colony-forming units [CFU] granulocyte-macrophage plus CFU-granulocyte, erythroid, monocyte, megakaryocyte) and represents a frequency of LTC-IC relative to all nucleated cells that is approximately 100-fold lower than that measured in normal marrow aspirate samples. Characterization studies showed most circulating LTC-IC to be small (low forward light scatter and side scatter), CD34+, Rh-123 dulled, HLA-DR+, and 4-hydroperoxycyclophosphamide-resistant cells, with differentiative and proliferative potentialities indistinguishable from LTC-IC in normal marrow. Isolation of the light-density, T-cell-depleted, CD34+, and either HLA-DR+ or Rh-123 dulled fraction of normal blood yielded a highly enriched population of cells that were 0.5% to 1% LTC-IC (~1,500-fold enriched beyond the light-density, T-cell-depletion step), a purity comparable to the most enriched populations of human marrow LTC-IC reported to date. However, purification of PB LTC-IC on the basis of these properties did not allow them to be physically separated from a substantial proportion (>30%) of the clonogenic cells in the same samples, in contrast to previous findings for LTC-IC and clonogenic cells in marrow. These studies show the presence in the blood of normal adults of a relatively small but readily detectable population of functionally defined, primitive hematopoietic cells that share properties with marrow LTC-IC, a cell type thought to have in vivo reconstituting potential. They also serve to emphasize the point that phenotypic characteristics previously associated with the most primitive hematopoietic cell types may not necessarily correlate with their developmental potential, underscoring the continuing importance of functional assays in future studies of early events in hematopoiesis.

The presence of primitive hematopoietic cells in adult peripheral blood (PB) has been recognized for three decades. Initial experiments showed that PB from a variety of species, including humans, was capable of protecting recipients from lethal doses of whole body irradiation by restoring blood cell formation from circulating donor cells. Subsequent studies led to the demonstration and quantitation of specific progenitor populations detected by colony assays. This prompted the evaluation of leukapheresis harvests as an alternative source of cells for therapeutic applications where autologous or allogeneic marrow transplants were not feasible. More recently, identification of strategies for increasing the concentration of clonogenic progenitors in the circulation has heightened interest in the potential of PB harvests for clinical treatment protocols requiring hematologic rescue.

Key to the future development of such protocols as well as to an improved understanding of factors that regulate stem cell mobilization, recruitment and marrow colonization is the availability of a quantitative assay for cells capable of reconstituting and sustaining hematopoiesis in transplant recipients. We have recently developed such an assay for murine hematopoietic stem cells, based on the demonstration of their competitive repopulating ability in vivo after injection into recipients at limiting dilution. These multipotent competitive repopulating units (CRU) are sustained in long-term cultures (LTC) of murine marrow where at least some may undergo multiple self-renewal divisions. These findings strongly suggest overlap or even identity of CRU with cells referred to as LTC-initiating cells (LTC-IC), the latter being defined by their ability to generate clonogenic progeny for periods in excess of 5 weeks under the same culture conditions that support CRU maintenance and proliferation.

The validity of the LTC-IC assay is dependent on the existence of a linear relationship between the endpoint measured (clonogenic cell output as assessed after 5 weeks of culture) and the number of LTC-IC in the original test suspension down to limiting numbers of LTC-IC. Previous analyses of the cellular input required to achieve a sustained output of clonogenic cells for 5 weeks or more indicated that, in the absence of added growth factors, two cell types were required: the LTC-IC itself, and a second ontologically unrelated “stromal” cell of the fibroblast-endothelial-adipocyte lineage that provides an essential support CRU maintenance and proliferation.

From the Terry Fox Laboratory, British Columbia Cancer Agency; and the Departments of Pathology, Medicine, and Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada.

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Address reprint requests to C.J. Eaves, PhD, Terry Fox Laboratory, BC Cancer Research Centre, 601 W 10th Ave, Vancouver, BC, Canada V5Z 1L3.

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supportive function. The latter proliferate in cultures initiated with unseparated cell suspensions of normal bone marrow (BM) and, if the initial number of BM cells present is relatively high (ie, > 10^6 cells/mL), will rapidly generate sufficient progeny to support the LTC-IC coexisting in the same original BM cell inoculum. However, to enable detection of LTC-IC in suspensions that lack supportive stromal cells or their precursors, as is the case for cells from normal adult blood, a separate source of supportive cells must be provided. We have previously shown that irradiated cells subcultured from the adherent layer of previously established normal marrow LTC or from a competent fibroblast cell line can be used for this purpose.

Under optimized assay conditions, LTC-IC in human marrow can be quantitated by limiting dilution analysis and have been shown to have several characteristics of quiescent cells. These characteristics include a relative insensitivity to 4-hydroperoxycyclophosphamide (4-HC), low retention of rhodamine-123 (Rh-123), small size, and low expression or absence of CD71 (the transferrin receptor) and HLA-DR. At least a proportion of the LTC-IC in normal adult human marrow show multipotentiality in the LTC system and maintain their numbers at levels comparable to CRU in analogous murine cultures. Several years ago, we reported that myeloid clonogenic cells were generated for at least 2 months in LTC cultures initiated by seeding T-cell–depleted light-density PB cells onto irradiated, pre-established allogeneic marrow-adherent layers, suggesting the normal presence of some LTC-IC in the circulation. In this study, we show that these cells can be quantitated using the same conditions and limiting dilution procedure originally developed and applied to the detection of LTC-IC in human marrow and that they also exhibit many of the same properties, as expected of a very primitive, quiescent hematopoietic cell.

MATERIALS AND METHODS

Cells. PB mononuclear cells were obtained with informed consent from normal volunteer blood donors as a byproduct of plateletphereses performed at the Vancouver General Hospital, Canada. Cells were further depleted of T cells by incubation with 2-aminoethylbromide isothiouronium-treated sheep red blood cells (RBCs) for 30 minutes at 4°C and subsequent isolation of the light density (ρ < 1.077 g/mL) fraction after centrifugation on Ficoll-hypaque (FH) as described previously. Random checking of this procedure showed that less than 2% of the recovered cells were CD2+ (T cells) by FACScan analysis. Normal BM aspirate cells were obtained with informed consent from normal donors of allogeneic marrow for transplantation. BM cells were either used directly, or after lysis of contaminating RBCs by brief exposure to ammonium chloride, or after centrifugation on FH as indicated.

Cultures. Cells from primary blood or BM samples or from LTC were assayed for erythroid (burst-forming units-erythroid [BFU-E]), granulopoietic (colony-forming units-granulocyte-macrophage [CFU-GM]), and multilineage (CFU-granulocyte-erythroid, monocyte, megakaryocyte [CFU-GEMM]) colony-forming cells in standard methylcellulose cultures containing 3 U/mL of human erythropoietin and 10% agar-stimulated human peripheral leukocyte-conditioned medium. This methodology and the criteria used for colony recognition have been described in detail previously. LTC-IC assays were initiated by seeding an aliquot of the test cell suspension into cultures containing irradiated (1,500 cGy) allogeneic marrow cells (3 × 10^3/cm^2) that had been subcultured from the adherent layer of previously established 2- to 4-week-old LTC. LTC-IC assay cultures were then fed weekly by replacement of half of the growth medium containing half of the nonadherent cells with fresh growth medium (0.5% medium supplemented with 10% foetal calf serum and 4-HC, 2.5 × 10^{-5} mol/L hydrocortisone sodium hemisuccinate, 12.5% horse serum, and 12.5% fetal calf serum [FCS]). In most experiments, LTC-IC assays were performed in cultures set up in 2.5 mL volumes in 35-mm tissue culture dishes, although for the limiting dilution assays, smaller, appropriately scaled down (0.1 mL) cultures were used as described previously. After a total of 5 weeks (unless specified otherwise), the nonadherent cells were removed, washed, and combined with cells harvested from the adherent fraction by trypsinization. These cells were then adjusted to a concentration suitable for plating in methylcellulose assays (to yield < 200 colonies per 1.1 mL assay culture). For a detailed description of the LTC-IC assay procedure, see Eaves et al. In the experiments reported here (unless specified otherwise), the number of clonogenic cells present in LTC harvested after 5 weeks (ie, the number of BFU-E plus CFU-GM plus CFU-GEMM present in both the nonadherent and adherent fractions at this time) was used to provide a quantitative, albeit relative, measure of the number of LTC-IC originally seeded into the LTC. However, as discussed in the Results, this number of clonogenic cells can be directly converted to an absolute number of LTC-IC simply by dividing by 4, because this is the average number of clonogenic cells calculated to be present in 5-week-old cultures per initial LTC-IC seeded.

Staining and flow cytometry. Cells were prepared for staining by resuspension in Hanks’ solution containing 2% FCS and 0.01% sodium azide (HFN). They were then incubated with 1 to 2 μg/mL of anti-HLA-DR-phycocerythrin (PE) (10^6 cells/mL) or Rh-123 at a final concentration of 0.1 μg/mL as described previously. In some cases, cells were stained with an anti-CD34 antibody (8G12) directly conjugated to PE or fluorescein isothiocyanate (FITC). Stained cells were sorted using a Becton Dickinson FACStarPlus (fluorescence-activated cell sorter [FACS]) equipped with an argon laser emitting at 488 nm (Becton Dickinson, Mountain View, CA). Fluorescence of Rh-123-, FITC-, and PE-labeled cells was measured using 530/30 and 575/26 band pass filters, respectively, after calibration of the FACS before each sort using 10 μm fluorescent beads. In some experiments, cells were gated according to their forward light scatter characteristics (FSC) and side scatter characteristics (SSC) to exclude most erythrocytes and granulocytes, as described previously. Cells appearing in this light scatter window (see Fig 4A, fractions I and II) constituted greater than 60% of the total light-density fraction of T-cell–depleted blood cells. Cells were collected after sorting in Hanks’ solution containing 50% FCS and were maintained at 4°C until plated.

RESULTS

Quantitation of LTC-IC in normal blood. In a first series of experiments, the number of clonogenic cells present after 5 weeks in LTC initiated with T-cell–depleted suspensions of normal PB mononuclear cells seeded onto pre-established, irradiated marrow adherent layers was found to be a linear function of the number of cells initially added over a 1,000-fold range of input cell numbers. Results for a representative experiment are shown in Fig 1. Three such dose response experiments also included a series of assay cultures (20 to 25 per point) that were seeded with limiting
numbers of LTC-IC (ie. ~1 LTC-IC per assay culture).

From the proportion of positive and negative assay cultures (containing ≥1 clonogenic cell each, or none, respectively), absolute frequencies of LTC-IC in the original test cell suspension were calculated using Poisson statistics (Fig 2).

Table 1. Quantitation of LTC-IC and Clonogenic Cells in Normal PB

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Concentration (per mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E</td>
<td>170 ± 20</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>2.9 ± 0.5</td>
</tr>
</tbody>
</table>

Values for individual patients were calculated by multiplying the progenitor frequency per 10^6 cells by the total nucleated cell recovery after both the T-cell-depletion and Ficoll density centrifugation steps and then again by the white blood cells per milliliter. Values shown are the mean ± SEM of data obtained from 23 different normal individuals.

From this value and a knowledge of the total number of clonogenic cells produced by a large number of cells of the same input suspension, the average 5-week output of clonogenic cells per LTC-IC in normal blood was calculated. This value was found to be 3.7 ± 1.2, which is similar to the value of 4.3 ± 0.4 that we reported for LTC-IC in normal BM. Bulk measurements of the 5-week clonogenic cell content of assay cultures initiated with T-cell-depleted blood samples from other normal adults could then also be used to derive absolute LTC-IC per milliliter values using this average clonogenic output per LTC-IC conversion factor. Table 1 shows the average concentration of LTC-IC in the PB calculated from values measured on 23 normal adults, together with the average concentration of circulating clonogenic cells (BFU-E plus CFU-GM plus CFU-GEMM) obtained for the same 23 samples. The derived value of approximately 3 LTC-IC/mL is approximately 75-fold lower than the concentration of circulating clonogenic cells both measured here (Table 1) and reported previously. Hence, the frequency of LTC-IC relative to other nucleated cells in the blood (~1 per 2 × 10^6) is approximately 100-fold lower than the frequency of LTC-IC relative to other cells in the BM.

**Phenotype of circulating LTC-IC.** The distributions of LTC-IC and clonogenic cells in various phenotypically defined subpopulations of the T-cell-depleted, light-density fraction of normal PB were then assessed. These were obtained using the FACS to separate cells on the basis of their light scattering properties, expression of CD34, HLA-DR, and Rh-123 uptake. As illustrated in Fig 3, even after removal of the T cells from the light-density fraction of leukapheresis samples, the frequency of cells expressing readily detectable levels of CD34 (as defined by the vertical gate shown in Fig 3B) was still very low (0.1% to 0.5%) as compared with the non-T-cell-depleted light-density fraction of normal marrow, where values of 1% to 4% are typically obtained, even using similarly stringent gating criteria. Most of the cells in the fraction defined as CD34+ expressed no or low levels of HLA-DR (Fig 3C) and had low SSC properties (Fig 3E and F). Cells with a CD34+ and HLA-DRlow phenotype (defined by the horizontal gate shown in Fig 3C) were found almost exclusively among the smallest light-density cells (low FSC, Fig 3E).

Figure 4 shows the distributions of LTC-IC and clonogenic cells observed when the total light-density T-cell-
A depleted fraction of PB cells was subdivided into three populations defined by their light scattering properties: I—low FSC, low SSC; II—intermediate to high FSC, low SSC; and III—all remaining cells (ie, open FSC, intermediate SSC). Although each gated population contained approximately equal numbers of cells, virtually all LTC-IC and most of the clonogenic cells were consistently found in the fraction containing the smallest cells (I). No LTC-IC and less than 5% of all clonogenic cells were found in fraction III. Therefore, in subsequent sorts, only cells in the low SSC fractions (I and II) were analyzed.

Figure 5 shows the results of functional assays performed on cells sorted both according to their expression of CD34 and HLA-DR. In this case, only CD34+ cells were assayed. These were then divided into HLA-DR^high and HLA-DR^low subpopulations using the gates shown in Fig 3C. In some experiments, CD34^+HLA-DR^low cells were further subdivided into an HLA-DR^- and an HLA-DR^+ population. No LTC-IC and very few directly clonogenic cells were detected in the CD34^+HLA-DR^high fraction. However, further subdivision of the remaining CD34^+HLA-DR^low cells did allow some differential separation of LTC-IC and
directly clonogenic cells, more of the latter (~40% v. ~10% LTC-IC) being found in the HLA-DR fraction. Table 2 shows the enrichment and recovery values obtained for LTC-IC and clonogenic cells in various HLA-DR subpopulations of the light scatter-gated, CD34+ fraction, as compared with the unstained, light-density, T-cell-depleted starting population in these experiments. Recovery of LTC-IC in the CD34+HLA-DRlow fraction was greater than 100% in all five experiments performed, suggesting that all circulating LTC-IC express readily detectable levels of CD34, as do those in normal BM.20 The isolation of a rare subpopulation of circulating cells defined by the same properties previously used to purify BM LTC-IC (ie, low-density, low forward light scatter, high expression of CD34, and low expression of HLA-DR), allowed a much greater enrichment (>1,000-fold beyond the light-density, T-cell-depletion step) of circulating LTC-IC to be routinely obtained. Thus, even though the initial frequency of LTC-IC in normal PB is much lower (on a per cell basis), the final purity of LTC-IC achievable from normal PB using these

Table 2. Frequency, Enrichment, and Recovery of LTC-IC and Clonogenic Cells in Various Subpopulations of the CD34+, T-Cell-Depleted, Light-Density Fraction of Normal PB Cells Defined According to Their Expression of HLA-DR

<table>
<thead>
<tr>
<th>Cell Type Evaluated</th>
<th>Source</th>
<th>Frequency (%)</th>
<th>Enrichment†</th>
<th>% Recovery‡</th>
<th>No. of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTC-IC</td>
<td>Unsorted cells</td>
<td>0.0022 ± 0.0004</td>
<td>—</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DRlow</td>
<td>0#</td>
<td>—</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DRlow</td>
<td>3.7 ± 1.1</td>
<td>1,930 ± 470</td>
<td>300 ± 40</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>DRlow</td>
<td>1.0 ± 0.5</td>
<td>540 ± 270</td>
<td>48 ± 36</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>DR++</td>
<td>2.8 ± 0.5</td>
<td>1,470 ± 340</td>
<td>280 ± 17</td>
<td>3</td>
</tr>
<tr>
<td>Clonogenic cells</td>
<td>Unsorted cells</td>
<td>0.11 ± 0.02</td>
<td>—</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DRlow</td>
<td>7.6 ± 0.4</td>
<td>65 ± 22</td>
<td>2.7 ± 1.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DR++</td>
<td>ND††</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>DR++</td>
<td>21 ± 2</td>
<td>240 ± 40</td>
<td>21 ± 6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DR++</td>
<td>15 ± 4</td>
<td>160 ± 40</td>
<td>38 ± 5</td>
<td>4</td>
</tr>
</tbody>
</table>

*Frequency of the cell type evaluated (LTC-IC or total clonogenic cells) relative to all nucleated cells in the population analyzed. (To convert the LTC-IC frequencies shown to absolute frequencies, divide by 4).
†Calculated by dividing the progenitor frequency per 10⁵ sorted cells by the progenitor frequency per 10⁵ unsorted, T-cell-depleted, light-density cells in each individual experiment, and then deriving the mean ± SEM of these values for the number of experiments performed.
‡Calculated by multiplying the percentage of cells retrieved in the fraction indicated by the corresponding calculated progenitor enrichment for each individual experiment (defined in footnote c), and then deriving the mean ± SEM of these values for the number of experiments performed.
§Measured as the total number of clonogenic cells present after 5 weeks (ie, ~4 x the absolute LTC-IC number)
∥Light-density, T-cell-depleted cells.
*As defined in Fig 3C.
#None detected; ie, less than 0.15. Enrichment and recovery values are therefore not calculated.
**Refers to a subpopulation of CD34+HLA-DRlow cells defined as fraction 2 in Fig 5A.
††Refers to a subpopulation of CD34+HLA-DRlow cells defined as fraction 1 in Fig 5A.
‡‡Not done as a separate measurement. DRlow recovery values can be inferred by adding together values for DR+ and DR−.
parameters (~0.5% to 1%, Table 2) was approximately the same as the best yet described for normal BM.\textsuperscript{21,27} Recovery of clonogenic cells in these same experiments appeared to be somewhat lower (Table 2), suggesting that some circulating clonogenic cells may have been excluded from the CD34\(^+\) population gated for in these studies, or that suboptimal plating efficiency of clonogenic cells may have been achieved when highly purified populations were assayed. Failure to detect additional clonogenic cells in the higher FSC/SSC fractions (II and III, Fig 4) due to potential inhibition of their colony-forming ability by the presence of increased numbers of monocytes was ruled out by mixing experiments (ie, no reduction of clonogenic cells detected when cells from fraction I were mixed with cells from fraction III in a 1:2 ratio; data not shown).

The results of combined staining for CD34 expression and Rh-123 uptake are shown in Fig 6. In this case, no difference was noted between circulating LTC-IC and clonogenic cells in terms of their distribution between the CD34\(^+\)Rh-123\(^{dull}\) and CD34\(^+\)Rh-123\(^{bright}\) fractions, with more than 80% of both being found in the Rh-123\(^{dull}\) fraction. This contrasts with normal BM, in which most of the LTC-IC are also Rh-123\(^{dull}\), but most of the clonogenic cells are Rh-123\(^{bright}\), thus allowing for their differential isolation by sorting according to this parameter.\textsuperscript{27} Nevertheless, the final purity of LTC-IC in the light-density, T-cell-depleted, CD34\(^+\)Rh-123\(^{dull}\) fraction of normal blood (data not shown) was similar to that obtained by selecting for HLA-DR\(^{low}\) cells (Table 2) or by application of the same criteria to BM.\textsuperscript{27} This reflects a similarly greater overall enrichment achieved with blood versus marrow using either HLA-DR expression or retention of Rh-123 as the final separation parameter.

4-HC sensitivities of circulating progenitors. Because normal circulating clonogenic cells were known to be a quiescent population\textsuperscript{43} and appeared phenotypically to be more similar to LTC-IC in either blood or BM than to the clonogenic cells found in the BM, it was of interest to compare the sensitivities of circulating clonogenic cells and LTC-IC to 4-HC, using the same type of treatment protocol that is in widespread clinical use for treating autologous marrow transplants. In this set of experiments, LTC-IC function (before or after exposure to 4-HC) was assessed in terms of the clonogenic cell content of assay cultures evaluated after 4 and 8 weeks (rather than after 5 weeks as in the experiments described above), because previous experiments had shown differences in LTC assays of BM samples for autologous transplants when these two time points were compared.\textsuperscript{25,26} Results for LTC-IC and clonogenic cells in normal PB and BM are shown in Fig 7. A dramatic difference in the effect of 30 minutes of exposure at 37°C to 100 \(\mu\)g/mL of 4-HC on the viability of clonogenic cells from blood and BM is apparent. Conversely, normal circulating clonogenic cells and LTC-IC appear to be similar to BM LTC-IC in their relative resistance to 4-HC. For LTC-IC from both sources, a slight increase in 4-HC resistance was noted for LTC-IC defined by the longer clonogenic cell output endpoint (ie, 8 weeks).

Differentiative potential expressed by circulating LTC-IC in LTC. Table 3 shows the relative proportions of BFU-E, CFU-GM, and CFU-GEMM in the total clonogenic population of 5-week-old LTC initiated with circulating LTC-IC of varying purities, and compares these with the relative numbers of these same types of clonogenic cells in the original blood samples. Data for unseparated and LTC-IC–enriched cell populations from normal BM obtained in previous studies\textsuperscript{30} are also shown in Table 3 for comparison. It can be seen that the differentiative behavior exhibited by LTC-IC in normal blood and BM is similar and is also not affected by the purity of the LTC-IC in the starting population. In both cases, a significant skewing towards the generation of CFU-GM by comparison to the number of

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**Fig 6.** (A) A representative histogram of CD34\(^+\), light-density, T-cell-depleted normal blood cells double-stained with Rh-123 and sorted into CD34\(^+\)Rh-123\(^{dull}\) and CD34\(^+\)Rh-123\(^{bright}\) fractions (fractions 1 and 2, respectively). The dark histogram in (A) shows the profile for unstained cells. (B) The mean ± SEM of the percentages of nucleated cells (■), clonogenic cells (□), and LTC-IC (■) in each sorted fraction (n = 3).
CFU-GM and BFU-E actually found in normal blood or BM was observed. To some extent this might be expected, because all stages of granulopoietic cell differentiation are supported in the LTC system, whereas erythropoiesis appears to be blocked at the stage of mature BFU-E production. As a result, this latter contribution to total BFU-E numbers in vivo is absent from LTC-derived populations.

**DISCUSSION**

We have previously described how the LTC system may be used to quantitate and characterize a very primitive cell in the BM of normal adults. Key to the interpretation of data obtained with this approach is the use of a competent feeder layer onto which the test cells are seeded so that the data obtained with this approach is the use of a competent feeder layer onto which the test cells are seeded so that the endpoint of hematopoietic activity measured several weeks later is determined solely and quantitatively by the number of primitive hematopoietic cells (LTC-IC) initially present. In addition, the duration of time allowed to elapse before assessment of the hematopoietic cell content of the culture and the level of differentiation of the hematopoietic cells measured are important. A minimum of 5 weeks is required for most input clonogenic cells to differentiate and disappear. Problems may also occur if quantitation of terminally differentiating granulocytes and macrophages are used as a read-out of input hematopoietic potential because the production of clonogenic cells and their subsequent differentiation into mature progeny in these cultures appear to be differently regulated. In the present study, we have explored the usefulness of the LTC-IC assay originally validated for human marrow cell suspensions for the assessment of primitive hematopoietic cells in normal PB. An additional requirement, noted previously, was the need to rigorously remove T cells from the mononuclear fraction of PB samples to be tested for their LTC-IC content to circumvent the otherwise frequent spontaneous emergence of rapidly growing Epstein-Barr virus (EBV)-transformed lymphocytes during the 4- to 8-week period required to complete the LTC-IC assay.

Our results show that LTC-IC can be reproducibly quantitated and found to be present, albeit at low levels, in normal adult blood. Similar findings have also recently been reported by Dooley and Law. We have further shown that normal circulating LTC-IC are undistinguishable from LTC-IC in normal BM in terms of the number and types of clonogenic progeny they produce and in terms of their expression of CD34, ability to retain Rh-123, and sensitivity to 4-HC. Two interesting differences, however, are the apparent smaller size (lower FSC) and lack of detectable expression of HLA-DR by most circulating LTC-IC. These findings reinforce the concept that "stromal" cell-mediated stimulation of long-term clonogenic cell production (>5 weeks) in vitro allows the detection of a functionally distinct primitive hematopoietic population that may, however, still include some biologic and, hence, phenotypic heterogeneity. Interestingly, although the concentration of circulating LTC-IC cells was found to be much lower (~75-fold) than the concentration of circulating clonogenic cells, the phenotypic characteristics of these functionally distinguished populations were found to be very similar, as has been previously shown for circulating clonogenic progenitors and the subset that produces colonies of blast cells capable of secondary colony formation in replating experiments.

Whether circulating LTC-IC represent a related or even

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**Table 3. Relative Proportions of Different Types of Clonogenic Cells Detected Before and After 5 Weeks in LTC (% of total)**

<table>
<thead>
<tr>
<th>Original Progenitor Source</th>
<th>No. of Samples</th>
<th>Clonogenic Cells*</th>
<th>LTC-IC†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BFU-E</td>
<td>CFU-GM</td>
</tr>
<tr>
<td>Light-density fraction of normal blood†</td>
<td>23</td>
<td>74 ± 3</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>LTC-IC-enriched fraction of normal blood‡</td>
<td>6</td>
<td>72 ± 5</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>Normal BM§</td>
<td>10</td>
<td>86 ± 3</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>LTC-IC-enriched fraction of normal BM¶</td>
<td>10</td>
<td>24 ± 5</td>
<td>75 ± 5</td>
</tr>
</tbody>
</table>

*Data shown are the mean ± SEM of proportional values for specific clonogenic cell types expressed as a percent of all clonogenic cells (ie, BFU-E plus CFU-GM plus CFU-GEMM) measured in standard short-term methylcellulose assays.
†Data shown are the mean ± SEM of proportional values for specific clonogenic cell types expressed as a percent of all clonogenic cells (ie, BFU-E plus CFU-GM plus CFU-GEMM) measured in methylcellulose assays of cells harvested from 5-week-old LTC.
‡Same samples as in Table 1.
§Data from LTC-IC in fraction 1 (CD34+DR−) in Fig 5 (n = 3) and fraction 1 (CD34+Rh-123[+]) in Fig 6 (n = 3).
¶Data for normal BM from previously published studies.20
overlapping subset of progenitors detectable by any of the previously described direct clonogenic cell assays remains unresolved. Additional experiments to look for the differential expression of other surface markers or growth factor receptors on circulating LTC-IC and different types of clonogenic cells may be helpful in this regard.

It may also be possible eventually to replace the adherent fibroblast-like “stromal” cells in the LTC-IC assay by a soluble source of the relevant factors they produce. Some success along these lines has been reported.47 Our own more recent studies have shown that the self-maintenance of LTC-IC can be fully retained when LTC-IC are cultured in the presence of Steel factor alone,48 although this can also be achieved when they are cocultured with murine SL/SL fibroblast feeders that do not contain the Steel gene.49 Such feeders, of course, also do not produce any species-specific human factors (eg, granulocyte-macrophage colony-stimulating factor [GM-CSF] and interleukin-3 [IL-3]), suggesting that another, as yet undefined factor(s) can support the maintenance and initial differentiation of human LTC-IC. Clearly, further studies will be required to establish the molecular identity of this factor(s), to determine whether a cellular mode of presentation is important, and to evaluate whether LTC-IC in marrow and blood are similarly regulated by such a factor(s).

Recently, we have shown that the LTC-IC assay can also be used to quantitate what appears to be a developmentally analogous primitive neoplastic (Ph1-positive) progenitor in patients with chronic myeloid leukemia (CML).50 The present studies thus also serve as an important baseline for comparison with these CML LTC-IC and will likely facilitate future analyses of abnormal properties of neoplastic stem cells in patients with other types of myeloproliferative or myelodysplastic clones.

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Characterization of primitive hematopoietic cells in normal human peripheral blood

C Udomsakdi, PM Lansdorp, DE Hogge, DS Reid, AC Eaves and CJ Eaves