Enhanced Detection, Maintenance, and Differentiation of Primitive Human Hematopoietic Cells in Cultures Containing Murine Fibroblasts Engineered to Produce Human Steel Factor, Interleukin-3, and Granulocyte Colony-Stimulating Factor

By D.E. Hogge, P.M. Lansdorp, D. Reid, B. Gerhard, and C.J. Eaves

To determine whether the sensitivity of the human long-term culture-initiating cell (LTC-IC) assay could be increased, we have evaluated a spectrum of different fibroblast cell lines for their abilities to influence the number of cells detectable as LTC-IC, to influence LTC-IC maintenance, and/or to influence LTC-IC differentiation into colony-forming cells (CFC) in cocultures containing various sources of LTC-IC. In a series of initial experiments with highly purified subpopulations of CD34+ cells from normal human marrow, no significant difference could be found between any of 3 different murine stromal fibroblast cell lines in terms of their support of either LTC-IC detection (CFC production) or maintenance (over a 6-week period), and all were equivalent to primary human marrow feeders (HMF). On the other hand, murine M2-10B4 fibroblasts engineered to produce high levels of both human granulocyte colony-stimulating factor (G-CSF) and interleukin-3 (IL-3; 190 and 4 ng/mL, respectively), either alone or mixed 1:1 with SI/Si fibroblasts engineered to produce high levels of soluble Steel factor (SF), with or without production of the transmembrane form of SF (60 and 4 ng/mL, respectively), stimulated the production of up to 20-fold more CFC in LTC of cells from normal human marrow, G-CSF–mobilized blood or cord blood when compared with parallel cocultures containing HMF. Limiting dilution analysis of the CFC output from all three sources of LTC-IC showed that most of this increase was due to an ability of the engineered feeders to increase the plating efficiency of the LTC-IC assay (~14-fold for marrow LTC-IC and ~4-fold for cord blood or mobilized blood LTC-IC). Analysis of the phenotype of these additionally recruited LTC-IC from marrow showed they had the same primitive CD34+CD45RA–CD71+ phenotype as conventionally defined LTC-IC. The limiting dilution studies also showed that the average number of CFC produced per LTC-IC was additionally and independently increased to yield values of 18 CFC per LTC-IC in marrow, 28 for LTC-IC in cord blood, and 25 for LTC-IC in G-CSF–mobilized blood. Replating of cells from primary LTC with different feeders into secondary LTC-IC assays containing the best combination of engineered feeders showed that LTC-IC maintenance could be significantly enhanced (up to 7-fold as compared with primary cocultures containing HMF). However, this enhancement was still not sufficient to amplify the number of LTC-IC present after 6 weeks above the input value. Thus, engineering murine fibroblasts to produce sufficient SF, G-CSF, and IL-3 can markedly enhance the detection as well as the maintenance in vitro of a very primitive population of human progenitor cells present in normal adult marrow, mobilized blood, and cord blood by providing the most sensitive assay conditions thus far described. The present findings also provide new evidence of biologic heterogeneity between different cell populations that can be operationally identified as LTC-IC, thus re-emphasizing the importance of limiting dilution analyses to distinguish between quantitative and qualitative effects on these cells.

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contact with stroma could be more effectively stimulated by such feeders than by factor addition to give equivalent concentrations in the medium.\textsuperscript{15} However, with the feeders generated in these earlier studies, only a modest increase in the number of colony-forming cells (CFC) generated after 5 weeks was obtained and the maintenance of their more primitive precursors (defined as long-term culture-initiating cells [LTC-IC]) was not substantially improved.\textsuperscript{1,2} It therefore seemed possible that a more significant enhancement of these responses might be obtained if other feeders could be identified or engineered that would produce higher levels of stimulatory factors. In the present report, we describe the results of such experiments in which stromal cell lines engineered to produce higher levels of different types and forms of human cytokines have yielded significant improvements in the human LTC-IC assay and have allowed functional differences between LTC-IC from different sources to be shown.

\section*{MATERIALS AND METHODS}

\textbf{Hematopoietic cells.} Normal human bone marrow from individuals donating marrow for allogeneic transplantation or from cadaveric donors, granulocyte colony-stimulating factor (G-CSF)-mobilized blood from patients undergoing leukapheresis for autologous blood progenitor cell collection, and cord blood cells were obtained with informed consent. Low-density cells (<1.077 g/mL) were isolated using a Ficoll/Hypaque gradient. Fluorescence-activated cell sorting (FACS)-purified cells were isolated as previously described\textsuperscript{4} after staining low-density marrow cells with anti-CD34 antibody (8G12) directly conjugated to Cy-5 in combination with anti-CD45RA (8d2) conjugated to R-phycoerythrin (PE) plus anti-CD7 (8G12) directly conjugated to Cy-5 in combination with anti-CD34 (OKT9) conjugated to fluorescin isothiocyanate (FITC) and propidium iodide (PhT; P-5264; Sigma, St Louis, MO). PI$^+$ cells with low forward and intermediate forward light scattering properties and low 90° light scattering properties and expressing high levels of CD34 were then sorted into fractions and isolated according to their expression of CD45RA and CD7.

\textbf{Feeders.} Human marrow fibroblast feeders (HMF) were generated from subcultured adherent layers of 2- to 4-week-old LTC and irradiated with 15 Gy of 250 Kvp x-rays before coculture with hematopoietic cells, as previously described.\textsuperscript{16} M2-10B4 cells are a Los Angeles, Los Angeles, CA). Transforming growth factor-\pi (TGF-\pi) cells derived from TGF-\beta knock-out mice and their +/+ controls\textsuperscript{20} were obtained from Dr S. Karlsson (National Institutes of Health, Bethesda, MD). All marrow feeders (including those described below) were irradiated with 80 Gy before being cocultured with human hematopoietic cells.

\textbf{Vectors.} The JZen-neo retroviral vector was constructed as previously described.\textsuperscript{20} The JZen-G-CSF-tkneo vector was constructed by placing the coding sequences for the human G-CSF cDNA (from Immunix Corp, Seattle, WA) 5’ of the tkneo cassette in the JZen-neo vector as described previously for an analogous interleukin-3 (IL-3) vector.\textsuperscript{21} The JZen-SF-tkneo vector was constructed in a similar fashion by inserting a 960-bp cDNA fragment containing the complete human SF coding sequence (from Dr K. Zsebo, formerly of Amgen, Thousand Oaks, CA) into the JZen-tkneo vector. The human IL-3 coding sequence was used to construct a vector with an alternative selectable marker, ie, hygromycin resistance. A 2,800-bp Xho I-HindIII fragment containing a hygromycin phosphotransferase-thymidine kinase fusion gene, termed hytk, linked to the cytomegalovirus promoter (from Dr S. Lupton, Immunex Corp)\textsuperscript{22} was subcloned into a modified JZen-1 vector termed JZen-1-NS (obtained from Dr D. Mager, Terry Fox Laboratory, Vancouver, British Columbia, Canada).\textsuperscript{27} Subsequently, the IL-3 cDNA fragment was subcloned 5’ of the hytk cassette in this retroviral plasmid.

To generate a plasmid to direct high-level expression of the transmembrane form of Steel factor (SF), an 820-bp fragment of human SF (hSF) engineered by deletion of exon 6 to lack the extracellular proteolytic cleavage site necessary for the release of soluble cytokine from the cell surface (from Dr F. Jacobsen, Amgen),\textsuperscript{28} was subcloned into the BCMGSNeo vector (from Dr H. Karasuyama, Basel Institute for Immunology, Basel, Switzerland).\textsuperscript{16} The BCMGSNeo vector is a bovine papilloma virus-based expression vector previously shown to direct stable, high-level expression of murine IL-2 when transfected into a variety of cell types.

The retroviral plasmids were transfected into the ecotropic retroviral packaging cell line, GP-E86 (obtained from Dr A. Bank, Columbia University, New York, NY).\textsuperscript{29} G418- or hygromycin-resistant transfected cells were then isolated, expanded, and assessed both for viral titer by the ability of their growth medium to generate drug-resistant NIH-3T3 cells and for the production of growth factor detectable by bioassay using appropriate growth factor-responsive cell lines.\textsuperscript{2,12} Cells producing viral titers of greater than 10⁶ colony-forming units (CFU)/mL were used to infect M2-10B4 cells or SI/SI fibroblasts.

\textbf{Generation of growth factor-producing feeders.} Cell-free growth medium was harvested from viral producer cells and, together with 8 \(\mu\)g/mL polybrene, added to subconfluent cultures of M2-10B4 cells and SI/SI fibroblasts. After 4 hours of incubation at 37°C, the virus-containing medium was replaced with growth medium. Two days later, infected cells were replated in growth medium containing G418 (GIBCO/BRL, Burlington, Ontario, Canada) for viruses containing the neo' gene (0.4 mg/mL [wt/vol] for M2-10B4 cells and 0.8 mg/mL for SI/SI fibroblasts) or medium containing hygromycin (0.02 mg/mL [wt/vol] for M2-10B4 cells and 0.125 mg/mL for SI/SI fibroblasts) for virus containing the gene encoding hygromycin resistance. When the selected cells had grown to confluence, the growth medium was tested for human IL-3 and SF bioactivity on M07e cells and for G-CSF activity on NFS-60 cells, as previously described,\textsuperscript{13} and for immunoreactive material by enzyme-linked immunosorbent assay (ELISA) using commercially available kits for these same three factors (R&D Systems, Minneapolis, MN). M2-10B4 cells producing both G-CSF and IL-3 were then infected with cells first with the JZen-G-CSF virus and by then infecting G418-resistant G-CSF-producing cells with the JZen-IL-3-hytk virus, with subsequent selection in hygromycin. SI/SI fibroblasts producing both the full-length SF molecule and IL-3 were made by infecting cells first with the JZen-SF-tkneo virus and then by infecting G418-resistant, SF-producing cells with the JZen-IL-3-hytk virus, with subsequent selection in hygromycin.

To generate SI/SI fibroblasts producing the transmembrane SF (tmSF) molecule, the BCMGSNeo-tmSF plasmid was transfected into the cells using calcium phosphate coprecipitation. Transfected cells were selected in G418. G418-resistant cells were grown to confluence and then analyzed for the expression of tmSF using flow cytometry. Cells were labeled with a monoclonal antibody to human SF (7H6, from Amgen)\textsuperscript{29} followed by incubation with FITC-conjugated sheep antimouse antibody (Becton Dickinson, Mountain View, CA). The labeled cells were analyzed using a FACSSort (Becton Dickinson) and the 5% most highly fluorescent cells were then sorted and grown to confluence in G418-containing medium. These cells were then tested for the production of SF by both bioassay\textsuperscript{21} and ELISA, as described above.

Mass cultures of these growth factor-producing fibroblasts were maintained and passaged as continuous cell lines. For use as feeders
in cocultures with human hematopoietic cells, 3 x 10^5 irradiated cells were seeded into 35-mm tissue culture dishes or 10^5 irradiated cells per well in 96-well plates (Nunc, Roskilde, Denmark).

**Cocultures and LTC-IC assays.** The human hematopoietic cells used as a source of LTC-IC were suspended in LTC medium (Myelocult; StemCell, Vancouver, British Columbia, Canada) to which freshly dissolved 10^{-7} mol/L hydrocortisone had just been added and then cocultured with specific irradiated feeders (or no feeders) as required by the experimental design. Cultures were maintained either at 33°C for 5 weeks or at 37°C for 6 weeks, as indicated, and fed in the interim with half-medium changes each week, as previously described. The total nonadherent and trypsinized adherent cell content of each culture was then harvested, the cells from both fractions were pooled, and appropriate aliquots were assayed for CFC and LTC-IC, as specified. In experiments with FACs-purified, CD34+ subpopulations of cells, 2,000 to 4,500 of these were cultured in 35-mm tissue culture dishes with or without feeders in a final volume of 2 mL of medium.

For the experiments shown in Table 6, the cells harvested from primary cocultures were assayed for LTC-IC in cultures that contained, as feeders, a 1:1 mixture of M2-10B4 cells producing IL-3 and high concentrations of G-CSF plus SI/SI cells producing IL-3 and SF. These secondary cocultures were then incubated for another 6 weeks at 37°C before being harvested for CFC assays. For limiting dilution analyses, low-density marrow, cord blood, or G-CSF–mobilized blood cells were plated at various cell concentrations (from 250 to 20,000 cells per well) on irradiated feeder cells in 96-well plates and the CFC content of each well was determined after 6 weeks at 37°C. The frequency of LTC-IC in the starting cell population and the average number of CFC generated per LTC-IC were calculated from these latter experiments, as previously described.

**CFC assays.** Cells to be assayed for burst-forming units erythroid (BFU-E), colony-forming units granulocyte/macrophage (CFU-GM), and CFU-granulocyte/macrophage/erythroid/megakaryocyte (CFU-GEMM) were plated in methylcellulose-containing medium supplemented with 50% fetal calf serum and 3 U/mL of human erythropoietin (StemCell). To this medium, 50 ng/mL human SF and 20 ng/mL each of human IL-3, IL-6, G-CSF, and GM-CSF were also added, except in some of the earlier experiments, in which, as indicated, 10% agar-stimulated human leukocyte-conditioned medium (LCM; StemCell) was used instead of recombinant growth factors. The SF and IL-6 were purified in our center from supernatants of COS cells transfected with the corresponding cDNAs; human IL-3 and GM-CSF were highly purified recombinant proteins obtained from Sandoz International (Basel, Switzerland) and G-CSF was from Amgen. CFC assay cultures were incubated for 2 to 3 weeks at 37°C and then erythroid, GM, and multilineage colonies were scored in situ. CFC numbers used to identify LTC-IC precursors represented the sum of all BFU-E, CFU-GM, and CFU-GEMM detected in harvests of LTC-IC assay cultures.

**RESULTS**

**Enhanced detection of LTC-IC–derived CFC using methylcellulose-containing recombinant growth factors.** In the original description of the assay for LTC-IC, cells from LTC were assayed for the presence of CFC in methylcellulose assays containing fetal calf serum and 10% agar-stimulated LCM. Using these CFC assay conditions, we showed that the average yield of CFC per LTC-IC was 4, regardless of the purity or source of the input LTC-IC or the type of fibroblast used as a feeder, although the CFC output from individual LTC-IC varied widely (from 1 to at least 30).

To determine the extent to which this value might be increased simply by substituting a cocktail of recombinant growth factors for the LCM in the methylcellulose assay medium, we first undertook experiments to identify a minimal cocktail that would give maximal detection of BFU-E, CFU-GM, and CFU-GEMM in both light-density and highly purified suspensions of human hematopoietic cells. A cocktail containing 50 ng/mL of SF and 20 ng/mL each of IL-3, IL-6, G-CSF, and GM-CSF was found to routinely give a twofold higher plating efficiency of each of these types of clonogenic progenitors than was obtainable with LCM, and further increases in the concentrations of any of these factors did not improve upon this (data not shown). Data illustrating this effect on freshly isolated CD34+ marrow cells are shown in Table 1 (the two far right columns). Thus, on average, the yield of CFC per LTC-IC from normal marrow assayed using HMF or unmanipulated M2-10B4 cells would be expected to be ~8, as was subsequently verified by limiting dilution analysis (see below). As is also shown in Table 1 for various phenotypically defined subsets of CD34+ cells, this cocktail of recombinant growth factors did not markedly change the distribution of different types of CFC in CD34+ subpopulations defined according to their level of expression of CD45RA or CD71. (Similar findings for LTC-IC are discussed below.)

**Survey of the supportive activity of different murine fibroblast cell lines in LTC initiated with human LTC-IC.** Some studies have suggested that different murine fibroblast cell lines may vary markedly in their ability to support the maintenance of very primitive murine hematopoietic cells, with the S17 line being found to be particularly effective in this regard. In addition, the documented importance of endogenously produced TGF-β in inhibiting the cycling of primitive CFC in human LTC suggested that it would be of interest to test fibroblasts from TGF-β1, −/− mice as feeders in the LTC system. However, when either of these types of stromal cells were tested as feeders, there was no significant difference in the number of CFC detectable after 6 weeks of their coculture with purified (CD34+ HLA-DR +) normal human marrow cells by comparison to control cocultures that contained HMF or fibroblasts obtained from TGF-β1, +/+ mice (the mean ± SD numbers of CFC in the test cultures relative to the numbers in cultures with HMF in three experiments being 1.7 ± 1.1, 0.5 ± 0.1, and 0.5 ± 0.6 for the S17, TGF-β1, +/−, and TGF-β1, −/− feeders, respectively).

**Characterization of murine fibroblast lines genetically engineered to produce high levels of human SF, IL-3, and G-CSF.** To test the possibility that feeders engineered to produce higher levels of IL-3 and G-CSF than had been previously achieved might provide better support for human hematopoietic cells and to determine whether the additional production of SF might enhance the effect of IL-3 and G-CSF, new generations of engineered murine fibroblast lines were isolated after transfer of the appropriate human cDNA clones, as described in the Materials and Methods. The retroviruses used for the transduction of M2-10B4 cells producing human G-CSF and IL-3 contained unique selectable markers linked to each cDNA, allowing for the isolation of doubly infected cells by simultaneous selection in G418 and
hygromycin. Medium conditioned by the feeders thus isolated contained 4 ng/mL and 190 ng/mL of human IL-3 and G-CSF, respectively. A similar strategy allowed SI/SI fibroblasts that generated 1 ng/mL and 4 ng/mL of human IL-3 and SF, respectively, to be isolated. However, it proved impossible to generate feeders producing more than 4 ng/mL of human SF using a variety of retroviral constructs and infection strategies. As an alternative approach, SI/SI fibroblasts were transfected with a bovine papilloma virus-based neo-containing expression vector, into which a cDNA encoding tmSF was placed with transfectants selected subsequently first in G418 and then for cell surface SF expression by flow cytometry using an anti-SF antibody. The engineered cells thus obtained expressed not only surface bound SF, but also bioactive SF into the medium for many weeks (to sustain levels of ~60 ng/mL). Release of soluble SF bioactivity from membrane-bound SF molecules lacking the proteolytic cleavage site encoded by exon 6 has been recently reported by others and, in the case of the murine SF, may result from cleavage at another site encoded by exon 7.

**Increased production of CFC in LTC containing genetically engineered feeders.** The ability of the human growth factor-producing murine feeder lines to enhance the output of CFC from FACs-purified CD34+ bone marrow cells was then tested in 6-week cocultures. Table 2 shows the results of a series of three such experiments in which CD34+ CD45RA-CD71+ cells were used as the source of the input LTC-IC. As expected from previous studies, the SI/SI-neo feeders and unmodified M2-10B4 cells were equivalent to the HMF controls. SI/SI feeders producing IL-3 and SF were superior to HMF in their ability to support CFC generation. The SI/SI feeders producing tmSF were similar in this respect, but not better. However, the M2-10B4 cells producing G-CSF and IL-3, either alone or when mixed with the SI/SI feeders producing SF ± IL-3, were able to further enhance the number of CFC produced (to yield 20-fold more CFC than cocultures with HMF).

### Table 1. Distribution of Different Types of Progenitors Among Various Subpopulations of CD34+ Human Marrow Cells Using Different Progenitor Assay Conditions

<table>
<thead>
<tr>
<th>Progenitor</th>
<th>Culture Conditions</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 1</th>
<th>Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTC-IC A</td>
<td></td>
<td>100</td>
<td>76</td>
<td>ND</td>
<td>2</td>
<td>ND</td>
<td>2</td>
<td>18</td>
<td>56</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>99.97</td>
<td>91</td>
<td>ND</td>
<td>2</td>
<td>0.03</td>
<td>7</td>
<td>93</td>
<td>123</td>
</tr>
<tr>
<td>BFU-E A</td>
<td></td>
<td>4</td>
<td>27</td>
<td>ND</td>
<td>3</td>
<td>96</td>
<td>70</td>
<td>53</td>
<td>34</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>4</td>
<td>55</td>
<td>ND</td>
<td>96</td>
<td>45</td>
<td>99</td>
<td>34</td>
<td>46</td>
</tr>
<tr>
<td>CFU-GM A</td>
<td></td>
<td>4</td>
<td>2</td>
<td>86</td>
<td>97</td>
<td>10</td>
<td>1</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>28</td>
<td>1</td>
<td>66</td>
<td>82</td>
<td>6</td>
<td>ND</td>
<td>48</td>
<td>74</td>
</tr>
<tr>
<td>CFU-GEMM A</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>48</td>
<td>123</td>
<td>86</td>
</tr>
</tbody>
</table>

*Abbreviation: ND, not detectable.
* One hundred percent equals the total number of each progenitor type in fractions I + II + III.
* Numbers of LTC-IC per 1000 CD34+ cells expressed as the total number of CFC detected in methylcellulose assays from 5- or 6-week-old LTC.

### Table 2. Relative Numbers of CFC Present in Coculture Containing Genetically Engineered Feeders

<table>
<thead>
<tr>
<th>Feeder</th>
<th>Fold Change in CFC Present in 6-Week-Old LTC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;0.01 ± 0.009†</td>
</tr>
<tr>
<td>SI/SI neo</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>M2-10B4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>M2-10B4 G + IL-3 (high)</td>
<td>19 ± 65†</td>
</tr>
<tr>
<td>SI/SI SF + IL-3</td>
<td>5 ± 3†</td>
</tr>
<tr>
<td>SI/SI Im SF</td>
<td>3 ± 3†</td>
</tr>
<tr>
<td>SI/SI tmSF:M2-10B4 G + IL-3 (1:1)</td>
<td>20 ± 10†</td>
</tr>
<tr>
<td>SI/SI SF + IL-3:M2-10B4 G + IL-3 (1:1)</td>
<td>20 ± 10†</td>
</tr>
</tbody>
</table>

* Cultures were initiated with CD34+“CD45RA-CD71+ human normal marrow cells. Results shown are the mean ± SD of results obtained in three experiments. Each test result has been expressed as a fold-change relative to control cocultures containing HMF.
† No CFC detected in any of the assays from all three experiments; the value shown indicates the limit of sensitivity of these measurements.
* P < .01 as compared with HMF (paired, two-tailed t-test).
$ P < .05 as compared with HMF (paired, two-tailed t-test).
Table 3. Limiting Dilution Analysis of LTC-IC Frequencies in Light-Density Normal Marrow Cells Cocultured With Different Murine Feeder Layers and Their Average CFC Output Values

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Frequency of LTC-IC*</th>
<th>CFC per LTC-IC</th>
<th>Frequency of LTC-IC*</th>
<th>CFC per LTC-IC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M2-10B4</td>
<td>Mix</td>
<td>M2-10B4</td>
<td>Mix</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>46</td>
<td>5</td>
<td>12</td>
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<tr>
<td>2</td>
<td>2</td>
<td>52</td>
<td>11</td>
<td>14</td>
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<td>3</td>
<td>1</td>
<td>10</td>
<td>8</td>
<td>24</td>
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<tr>
<td>4</td>
<td>8</td>
<td>66</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>Mean</td>
<td>3</td>
<td>43</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>SD</td>
<td>±3</td>
<td>±24</td>
<td>±3</td>
<td>±6</td>
</tr>
</tbody>
</table>

* Per 10⁶ light-density normal marrow (input) cells.
† CFC per LTC-IC were calculated as average values by dividing the total number of CFC produced in all of the limiting dilution cultures plated by the number of calculated LTC-IC used to initiate those cultures for each culture condition tested.
‡ Mix refers to a 1:1 mixture of M2-10B4 cells producing 190 ng/mL G-CSF and 4 ng/mL of IL-3, with S/I cells producing 4 ng/mL of soluble SF in addition to IL-3.

To determine whether the increased numbers of CFC detected in LTC containing the growth factor-producing feeders might be due to the enhanced survival or amplification of pre-existing clonogenic cells, the phenotype of the cells responsible for their generation was analyzed. The results of such experiments are shown in Table 1, in which low-density marrow cells were sorted into different subpopulations of CD34⁺ cells, and then aliquots from each of these subpopulations were plated directly in clonogenic assays using agrastimulated LCM or recombinant cytokines as a source of growth factors as well as in LTC using either HMF or the mixed growth factor-producing feeders. As can be seen from the results obtained, the cells detected as LTC-IC in assays that contained the mixed feeders were, like those identified in the conventional assay, found exclusively in the CD45RA⁻CD71⁻ fraction of CD34⁺ marrow cells.

Increased detection of LTC-IC using genetically engineered feeder cells as shown by limiting dilution analysis. Although the phenotype of the cells producing the increased numbers of CFC detectable in 6-week-old cocultures containing the mixed feeders appeared to be unchanged, this result would not discriminate between an increase in the average output of CFC from each LTC-IC initially plated and an increase in the number of initially seeded cells that were able to generate CFC 6 weeks later. To distinguish between these possibilities, light-density bone marrow cells were cocultured at limiting dilution so that the LTC-IC frequencies could be determined independently of their CFC output using Poisson statistics. In these experiments, unmodified M2-10B4 cells were used as a control because they had been previously shown to give results equivalent to HMF.

The results of four experiments are shown in Table 3. It can be seen that most of the increased CFC production observed in bulk cultures containing a 1:1 mixture of M2-10B4 cells producing G-CSF and IL-3 with S/I cells producing SF plus IL-3 is attributable to an increase in the frequency of cells detectable as LTC-IC under these conditions (~14-fold higher than with unmodified M2-10B4 cells). However, the number of CFC produced per LTC-IC was also slightly (3-fold) higher in assays containing this mixed engineered feeder. Thus, both the detection of LTC-IC and their yield of progeny CFC can be significantly enhanced when fibroblasts constitutively producing high levels of SF, IL-3, and G-CSF are used as feeders (P < 0.05, paired, two-tailed t-test).

Similar limiting dilution experiments were also performed using light-density cord blood cells and unseparated G-CSF-mobilized peripheral blood cells as the input populations. As shown in Table 4, in each of three such experiments with cord blood, the frequency of LTC-IC detected was higher on the engineered feeders producing the three human growth factors than on unmodified M2-10B4 cells. However, in this case, the increase was only fourfold and the accompanying increase in the number of CFC produced per LTC-IC, although significant (P < 0.05, paired, two-tailed t-test), was also more modest (from a mean of 17 to 28).

Table 4. Limiting Dilution Analysis of LTC-IC Frequencies in Light-Density Cord Blood Cells Cocultured With Different Murine Feeder Layers and Their Average CFC Output Values

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Frequency of LTC-IC*</th>
<th>CFC per LTC-IC</th>
<th>Frequency of LTC-IC*</th>
<th>CFC per LTC-IC</th>
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<tr>
<td></td>
<td>M2-10B4</td>
<td>Mix</td>
<td>M2-10B4</td>
<td>Mix</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>62</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>26</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>5</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td>Mean</td>
<td>7</td>
<td>31</td>
<td>17</td>
<td>28</td>
</tr>
<tr>
<td>SD</td>
<td>±6</td>
<td>±25</td>
<td>±2</td>
<td>±2</td>
</tr>
</tbody>
</table>

* Per 10⁶ light-density cord blood (input) cells.
† Calculated as described in the legend to Table 3.
‡ Mix is the same as described in the legend to Table 3.

Table 5. Limiting Dilution Analysis of LTC-IC Frequencies in G-CSF-Mobilized Peripheral Blood Cocultured With Different Murine Feeder Layers and Their Average CFC Output Values

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Frequency of LTC-IC*</th>
<th>CFC per LTC-IC</th>
<th>Frequency of LTC-IC*</th>
<th>CFC per LTC-IC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M2-10B4</td>
<td>Mix</td>
<td>M2-10B4</td>
<td>Mix</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>19</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>24</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>37</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>Mean</td>
<td>6</td>
<td>27</td>
<td>9</td>
<td>25</td>
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<tr>
<td>SD</td>
<td>±4</td>
<td>±3</td>
<td>±2</td>
<td>±5</td>
</tr>
</tbody>
</table>

* Per 10⁶ nucleated unseparated peripheral blood (input) cells.
† Calculated as described in the legend to Table 3.
‡ Mix is the same as described in the legend to Table 3.
Detection of LTC-IC, cells from 6-week-old primary cocul-
ferent types of feeders, particularly those that enhanced the
CFC detected were restricted to the granulocyte-mac-
rophage progenitors generated was not different. In cultures
of marrow, cord blood, or mobilized blood, at least 95% of
the CFC detected were restricted to the granulocyte-macro-
phage lineage regardless of the feeder used.

Improved LTC-IC maintenance in cocultures containing engineered feeders. To evaluate whether the maintenance
of LTC-IC might also be affected by their coculture on dif-
frent types of feeders, particularly those that enhanced the
detection of LTC-IC, cells from 6-week-old primary cocul-
tures were replated into secondary LTC-IC assay cultures.
Because of the increased sensitivity of LTC-IC detection in
assays that used the mixed engineered feeders and methylcel-
lulose CFC assays containing recombinant growth factors,
these conditions were used to quantitate the input LTC-IC
and the number recovered from the various cocultures 6
weeks later. As shown in Table 6, only 0.1% of the input
LTC-IC number was detectable after 6 weeks, unless some
type of feeder layer was present. Even in the presence of
HMF, unmanipulated M2-10B4 cells, or S/SI Sneo cells, the
number of LTC-IC remaining after 6 weeks was less than
10% of the input value. Similar results were obtained with
feeders in which the M2-10B4 cells producing G-CSF and
IL-3 were mixed with the SI/SI cells producing SF ± IL-3.
On the other hand, in the presence of only the SI/SI cells
producing SF ± IL-3, the maintenance of the LTC-IC popu-
lation was enhanced (~4-fold and 7-fold, respectively) by
comparison to cultures containing HMF. However, despite
this increase, the number of LTC-IC detectable after 6 weeks
was still lower than the input value (41%). G-CSF + IL-3-
producing M2-10B4 cells also appeared to improve LTC-
IC maintenance, but this effect was more variable (only seen
in 2 of the 3 experiments) and did not reach statistical sig-
ificance.

**DISCUSSION**

Hematopoietic cells, which initiate long-term myeloid hematopoiesis when cocultured with primary marrow adherent
cell layers, appear to represent a very primitive progenitor
cell type with a number of properties that are shared with
murine long-term in vivo repopulating cells. Our original
studies on the detection, proliferation, and differentiation of
human LTC-IC showed such cells to be present in normal
human marrow at an average frequency of approximately 1
in 20,000 cells, of which at least a subset could be shown
to have multilineage potential and to give rise to as many
as 30 clonogenic cell progeny. However, the mean number of
CFC generated per LTC-IC was only 4, the majority
(90%) of which were CFU-GM.

Parallel studies showed the constitutive or induced pro-
duction by LTC adherent layers of a number of candidate
positive regulators, including G-CSF, GM-CSF, IL-1β, IL-
6, and SF. However, evidence was also obtained to indicate
that the stromal adherent layer, in addition to having a sup-
portive function, is an important source of factors that block
the proliferation of primitive CFC and that growth may be
determined by the net balance achieved between such oppos-
ing activities. 

Table 6. Maintenance of LTC-IC Numbers for 6 Weeks in Cocultures Containing Various Genetically Engineered Feeders

<table>
<thead>
<tr>
<th>Feeder</th>
<th>Fold Change</th>
<th>% of Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.02 ± 0.04</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>HMF</td>
<td>1</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>S/SI Sneo</td>
<td>1 ± 0.3</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>M2-10B4</td>
<td>0.6 ± 0.7</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>M2-10B4 G + IL-3</td>
<td>4 ± 5</td>
<td>23 ± 30</td>
</tr>
<tr>
<td>S/SI SF + IL-3</td>
<td>7 ± 3*</td>
<td>41 ± 26</td>
</tr>
<tr>
<td>S/SI tmSF</td>
<td>4 ± 2*</td>
<td>23 ± 14</td>
</tr>
<tr>
<td>M2-10B4 G + IL-3:S/SI tmSF (1:1)</td>
<td>2 ± 2</td>
<td>9 ± 11</td>
</tr>
<tr>
<td>M2-10B4 G + IL-3:S/SI IL-3 + SF (1:1)</td>
<td>1 ± 1</td>
<td>6 ± 6</td>
</tr>
</tbody>
</table>

Cultures were initiated with CD34+CD45RA-CD71+ human marrow cells. Values are the mean ± SD of results from three experiments.

* P < .05 as compared with HMF (paired, two-tailed t-test).

Thus, although the LTC system has been useful because of
its apparent ability to support the selective long-term prolif-
eration of very primitive types of human hematopoietic cells,
the conditions prevailing in conventional LTC must be
grossly suboptimal by comparison to those operating in stro-
mal-free cultures in which clonal populations of greater than
10⁶ cells can be readily obtained or in vivo in which the
generation of clonal populations of greater than 10⁷ cells has been documented.

Several avenues of investigation have subsequently been
pursued to attempt to understand and improve the stimulation
of LTC-IC in vitro. One has been to prevent these cells from
making direct contact with the fibroblast-containing feeder
layer. This approach, together with the use of exogenous
factor addition, has enabled a net amplification in both CFC
and LTC-IC numbers (relative to input values) to be achieved
under both static⁶ and stirred suspension conditions. Exhaus-
tion of key components of the growth medium and/or
accumulation of negative factors are examples of other
potentially limiting culture parameters that can be circum-
vented by appropriate adjustment of the rate of medium
exchange and/or the use of highly purified starting popula-
tions of LTC-IC together with suitable cytokine combina-
tions. The approach that we have focussed on in this
study has involved an evaluation of the potential of variously
modified fibroblast feeder layers, which would be anticipated
either to decrease production of an endogenously derived
inhibitor or to increase the constitutive production of stimu-
lators of LTC-IC. Interestingly, we found that simple elimi-
nation of fibroblast-derived TGF-β, was insufficient to sig-

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These experiments showed positive effects on both of these and a corresponding fourfold increase for LTC-IC from cord blood or mobilized blood (to producing both SF and IL-3 plus M2-10B4 cells). Purified CD34+ marrow cells suggest this to be the enhancement of CFC output after 6 weeks (ie, SYS1 cells). For one of the feeder combinations that gave maximal increase (SF, IL-3, and SF) were able to amplify significantly over that seen with HMF or control murine fibroblasts but were somewhat less effective in this regard than the G-CSF-producing M2-10B4 cells. It is possible that other characteristics unique to M2-10B4 cells may specifically enhance the ability of constitutively produced G-CSF to support an increased CFC production from LTC-IC. However, one obvious possibility is that G-CSF at sufficient concentrations is particularly potent in this regard. In fact, recent experiments with soluble factors in short-term suspension cultures of highly purified CD3+CD38− marrow cells suggest this to be the case.

For one of the feeder combinations that gave maximal enhancement of CFC output after 6 weeks (ie, SI/S1 cells producing both SF and IL-3 plus M2-10B4 cells producing IL-3 plus high levels of G-CSF), limiting dilution experiments were performed to allow effects on LTC-IC plating efficiency and CFC output per LTC-IC to be distinguished. These experiments showed positive effects on both of these endpoints. Thus, using these feeders, an 14-fold increase in the plating efficiency of LTC-IC from normal marrow and a corresponding fourfold increase for LTC-IC from cord blood or mobilized blood (to 1 per 3,000 light-density cells for both marrow and cord blood and 1 per 4,000 for mobilized blood cells) was seen. From these data it is clear that cells that were not detectable as LTC-IC under the original assay conditions described using HMF27 were recruited to LTC-IC maintained under LTC conditions may be an inadequate reflection of their in vivo capabilities. Thus, the effects obtained with these feeders could not be attributed exclusively to differences in SF presentation in a cell-exposed medium, presumably due to extracellular cleavage of the molecule at an alternative site outside of the missing sixth exon.20

Thus, it seems unlikely that these new LTC-IC are drawn from a pre-existing LTC-IC population. Such an interpretation is also supported by the recently shown inability of these cells to proliferate in methylcellulose.3 Thus, we favor the interpretation that the LTC-IC that require growth factor feeders for their detection represent a heretofore functionally silent population of LTC-IC that can express this potential within 6 weeks only under more potent conditions of stimulation. The fact that marked increases were seen in the number of LTC-IC detected using the growth factor-producing feeders in assays of cells from cord and mobilized blood as well as marrow would suggest that LTC-IC from these sources have also been previously under estimated. In addition to an increased frequency of LTC-IC in all 3 cell sources examined, the CFC output per LTC-IC was also increased approximately twofold in each case (>2-fold for marrow and mobilized blood LTC-IC and slightly <2-fold for LTC-IC from cord blood) in assays containing the growth factor-producing feeders. This finding, together with the improved detection of the CFC produced through the substitution of recombinant growth factors for agar LCM in the methylcellulose assays, resulted in new values for the average output of CFC per LTC-IC of 18, 28, and 25 for marrow, cord blood, and mobilized blood progenitors, respectively. Thus, both the functional properties as well as the frequency of LTC-IC can vary greatly depending on the assay conditions. In addition, it seems likely that, despite the improvements introduced here, the developmental potential shown by LTC-IC maintained under LTC conditions may be an inadequate reflection of their in vivo capabilities.
containing SF-producing feeders, the number of LTC-IC present after 6 weeks at 37°C was still only approximately half of the input number. Attempts to further enhance the maintenance of LTC-IC by combining two feeder types were unsuccessful and, in some instances, actually had a negative effect. Thus, LTC using mixed feeders producing SF, IL-3, and G-CSF, which were most successful in enhancing expression of the differentiative capacity of LTC-IC (the number of CPC produced per LTC-IC), may have achieved that effect at the expense of the maintenance of the most primitive progenitors. Better expansion of LTC-IC has recently been achieved in stirred suspension cultures containing soluble cytokines, 13 in stroma noncontact cultures, 14 in perfusion bioreactors, 15 and, most dramatically, in suspension cultures of highly purified LTC-IC stimulated by high concentrations of Flt-3-ligand, SF, and IL-3. 16 Thus, contact between stromal cells and hematopoietic cells may be a major barrier to achieving significant LTC-IC amplification. Nevertheless, the use of engineered feeders still remains an attractive strategy due to the potential of this approach to bypass any need for the continuous addition of exogenous cytokines. Moreover, given the availability of evolving technologies for inducible expression of transferred genes, 17 the creation of engineered feeders might also lend itself to the additional testing of genetically defined differences in sup-
portive stromal cells may allow further elucidation of critical mechanisms used by the hematopoietic microenvironment to regulate the proliferation and differentiation of primitive hematopoietic progenitors.

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