Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1470–1474, February 1996
Medical Sciences

Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) *in vitro* and their expansion in defined medium

(stem cells/hematopoiesis/amplification)

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Communicated by Irving M. London, Harvard University-Massachusetts Institute of Technology, Cambridge, MA, November 2, 1995

ABSTRACT **A major goal of experimental and clinical hematology is the identification of mechanisms and conditions that support the expansion of transplantable hematopoietic stem cells. In normal marrow, such cells appear to be identical to (or represent a subset of) a population referred to as long-term-culture-initiating cells (LTC-ICs) so-named because of their ability to produce colony-forming cell (CFC) progeny for ≥5 weeks when cocultured with stromal fibroblasts. Some expansion of LTC-ICs *in vitro* has recently been described, but identification of the factors required and whether LTC-IC self-renewal divisions are involved have remained unresolved issues. To address these issues, we examined the maintenance and/or generation of LTC-ICs from single CD34$^+$ CD38$^-$ cells cultured for variable periods under different culture conditions. Analysis of the progeny obtained from cultures containing a feeder layer of murine fibroblasts engineered to produce steel factor, interleukin (IL)-3, and granulocyte colony-stimulating factor showed that ≈20% of the input LTC-ICs (representing ≈2% of the original CD34$^+$ CD38$^-$ cells) executed self-renewal divisions within a 6-week period. Incubation of the same CD34$^+$ CD38$^-$ starting populations as single cells in a defined (serum free) liquid medium supplemented with Flt-3 ligand, steel factor, IL-3, IL-6, granulocyte colony-stimulating factor, and nerve growth factor resulted in the proliferation of initial cells to produce clones of from 4 to 1000 cells within 10 days, ≈40% of which included ≥1 LTC-IC. In contrast, in similar cultures containing methylcellulose, input LTC-ICs appeared to persist but not divide. Overall the LTC-IC expansion in the liquid cultures was 30-fold in the first 10 days and 50-fold by the end of another 1–3 weeks. Documentation of human LTC-IC self-renewal *in vitro* and identification of defined conditions that permit their extensive and rapid amplification should facilitate analysis of the molecular mechanisms underlying these processes and their exploitation for a variety of therapeutic applications.**

Adult hematopoietic tissues contain a small population of totipotent cells, each with the capacity to generate very large numbers of mature progeny (>10^{13} cells) over prolonged periods of time (1). The development of assays suitable for the quantitation and distinction of these cells from other primitive hematopoietic cells with more restricted proliferative and/or differentiation potentialities has thus posed a significant challenge to the field. In the murine system, it has been shown that transplantable totipotent hematopoietic stem cells with long-term *in vivo* repopulating ability can be quantitated by an appropriate limiting dilution assay (2). However, an analogous procedure is not yet available for human stem cells and surrogate methods are, therefore, required. The long-term-culture-initiating cell (LTC-IC) assay that detects cells that can generate myeloid clonogenic cell (colony-forming cell, CFC) progeny in long-term cultures for a minimum period of 5 weeks (3) appears to be useful in this regard since many of the LTC-ICs thus identified in both human and mouse marrow share a unique and rare phenotype characteristic of transplantable murine *in vivo* repopulating cells (4). Most attempts to identify culture conditions able to support a net expansion of LTC-ICs (or *in vivo* repopulating cells in the murine system) have to date been disappointing (5–7), in spite of considerable success with later types of progenitor cells (8–10). Nevertheless, the ability of some transplantable murine stem cells to be amplified in the presence of marrow stromal fibroblasts both *in vitro* (11) and *in vivo* (12) is well documented. It is, therefore, widely assumed that the self-renewal of human totipotent hematopoietic cells can also occur under appropriate conditions. Recently, there have been two reports of limited net increases in the LTC-IC content of cultures initiated with adult human marrow cells (7, 13). In the studies described herein, we investigated the LTC-IC output from individual primitive cells stimulated to proliferate *in vitro* under different conditions. To obtain a starting population that was sufficiently enriched in primitive cells to make such an analysis feasible, we isolated the light-density CD34$^+$ CD38$^-$ fraction of normal human marrow cells (14). The results support the concept that LTC-ICs and CFCs are distinct (nonoverlapping) progenitor populations and document the potential of LTC-ICs to execute self-renewal divisions *in vitro*. In addition, these studies have shown that LTC-IC numbers can be rapidly amplified >50-fold within a 3- to 5-week time frame in low-density cultures containing an appropriate cytokine mixture in the absence, but not in the presence, of methylcellulose.

MATERIALS AND METHODS

Purification of Bone Marrow Cells. Light-density (<1.077 g/cm^3) bone marrow cells were isolated from cadaveric organ donor samples by using Ficoll/Paque (Pharmacia). The cells were then washed once in PBS containing 2% (vol/vol) fetal calf serum (StemCell Technologies, Vancouver, BC) and resuspended in Hanks' Hepes-buffered salt solution containing 2% fetal calf serum and 0.1% sodium azide (HFN) for subsequent staining. CD34$^+$ CD38$^-$ cells were isolated by flow cytometry either in a single step from cells labeled simultaneously at ≤10^7 cells per ml for 30 min at 4°C with predetermined optimal concentrations of fluorescein isothiocyanate-coupled anti-CD34 and phycoerythrin-coupled anti-CD38

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Abbreviations: LTC-IC, long-term-culture-initiating cell; CFC, colony-forming cell; IL, interleukin; CSF, colony-stimulating factor; G, granulocyte; M, macrophage; SF, steel factor; β-NGF, β nerve growth factor.

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(Leu17-PE, Becton Dickinson), or in a two-step procedure in which the $CD34^+$ $CD45RA^-$ $CD71^-$ fraction was first isolated, the cells then restained with phycoerythrin-coupled anti-CD38, and $CD34^+$ $CD38^-$ cells were selected as described (14). Propidium iodide (Sigma) at 2 μg/ml was added to the second wash in HFN prior to sorting and the cells were finally resuspended in HF (no azide) before sorting with a $FACStar^+$ (Becton Dickinson) equipped with a 5-W argon laser, a helium/neon laser, and an automatic cell deposition unit. Cells with low to intermediate forward light scattering properties, low side scattering properties, and a $CD34^+$ $CD38^-$ phenotype were either collected in bulk or distributed individually into flat- or round-bottomed wells in Nuncleon 96-well plates (Nunc) as indicated.

Progenitor Assays. LTC-IC assays were performed as described (14) by using a slight modification of the original procedure (3). Briefly, test cells were suspended in long-term culture medium (MyeloCult, from StemCell) to which freshly dissolved hydrocortisone sodium hemisuccinate (Sigma) was added just prior to use to give a final concentration of 10^{-6} M and then cocultured on irradiated (80 Gy) feeder layers of mouse fibroblasts engineered to produce human interleukin (IL) 3 (4 ng/ml), human granulocyte colony-stimulating factor (G-CSF) (190 ng/ml), and human steel factor (SF) (4 ng/ml) for 6 weeks at 37°C with weekly changes of half the medium. At the end of the 6-week LTC-IC assay period, nonadherent cells were removed from the cultures, combined with the corresponding trypsinized adherent cells, washed, and assayed for CFCs in methylcellulose medium (MethoCult, StemCell) containing 30% fetal calf serum, erythropoietin (3 units/ml), SF (50 ng/ml), and IL-3 (20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml), and granulocyte/macrophage colony-stimulating factor (GM-CSF) (20 ng/ml). These cultures were incubated at 37°C and colonies were scored 2–3 weeks later. In some cases, only part of the harvests were assayed for CFCs and the remaining cells were used for other purposes as indicated.

Single-Cell Suspension Cultures. Single $CD34^+$ $CD38^-$ cells were cultured at 37°C in round-bottomed wells of 96-well plates in 100 μl of Iscove's medium supplemented with bovine serum albumin (20 mg/ml), insulin (10 μg/ml), and transferrin (200 μg/ml) (all from StemCell), instead of serum, plus 10^{-4} M 2-mercaptoethanol and the following cytokines: SF (100 ng/ml), Flt-3 ligand (100 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml), and β-nerve growth factor (β-NGF, R&D Systems; 5 ng/ml). At the end of 10 days, all of the cells from each well were harvested individually and all or part were assayed for LTC-ICs as described above. Remaining cells were transferred to new wells in 100 μl of fresh serum-free medium containing the same cytokines and cultured for an additional 1–3 weeks at 37°C prior to performing LTC-IC assays.

RESULTS

LTC-IC and CFC Content of the $CD34^+$ $CD38^-$ Fractions of Normal Human Bone Marrow. The average frequency of LTC-ICs in the $CD34^+$ $CD38^-$ cells used was found to be at least 10% (see *Results* for week-6 LTC-IC assays in Table 1 and LTC-IC input in Table 2). This was determined by plating single $CD34^+$ $CD38^-$ cells into individual flat-bottomed wells of 96-well plates containing preestablished feeder layers and then assessing the number of CFCs present 6 weeks later. Aliquots of the same $CD34^+$ $CD38^-$ starting populations were also plated directly into methylcellulose cultures (see *Results* for CFC input in Table 2). Very few $CD34^+$ $CD38^-$ cells were able to form visible colonies in methylcellulose and, in every experiment, the proportion of cells detectable as CFCs was much lower than the proportion of cells detectable as LTC-ICs. Since many of the same cytokines (i.e., SF, IL-3, and G-CSF) were present in the assays used to detect CFCs and LTC-ICs in these studies, this suggests that other features of the two assays allow largely, if not completely, distinct cell types to be detected.

To investigate the possibility that LTC-ICs may undergo self-renewal divisions *in vitro* and to further examine the potential relationships of LTC-ICs and CFCs, two additional types of experiments were performed. In the first, a series of LTC-IC assay cultures were initiated with single $CD34^+$ $CD38^-$ cells and then 6 weeks later half of the cells were plated in methylcellulose assays to identify those wells in which CFCs had been generated (and hence those that had been initially seeded with an LTC-IC). The other half of each well was transferred well by well to new (secondary) LTC-IC assay cultures. These were then maintained for a further 6 weeks before being harvested and assayed individually to determine the number and type of CFCs that were present. As shown in Table 1, the proportion of input LTC-ICs (identified by their ability to produce ≥1 CFC detectable at the end of the first 6 weeks) that also gave rise to daughter LTC-ICs (as shown by their ability to produce ≥1 CFC detectable after another 6 weeks) varied from 14% to 37% in two of the three such experiments performed and was, therefore not surprisingly, lower than the 20% limit of detectability in the other experiment. Moreover, there was no obvious change in the number of CFCs produced per well after 12 as compared to 6 weeks (data not shown) as shown previously for LTC-ICs measured in cultures initiated with larger numbers of cells (3, 15). Interestingly, in all three experiments, a significant proportion of the input cells that could not be detected initially as LTC-ICs (using a 6-week endpoint to detect the generation of progeny CFC) did eventually give rise to CFCs that were detected at the end of 12 weeks (in the secondary long-term culture). In fact, these latter results show that assessment of CFC generation after an interval of 6 weeks may fail to detect as many as 40% of all stromal-cell-responsive CFC precursors present in the $CD34^+$ $CD38^-$ fraction of normal marrow, even when these are cultured in direct contact with fibroblast feeders engineered to constitutively produce high levels of SF, IL-3, and G-CSF. When both 6- and 12-week endpoints of CFC generation were used to define LTC-ICs, at least 20% of the $CD34^+$ $CD38^-$ marrow population could be accounted for. Presumably, this proportion would increase further if CFC outputs at time points in between 6 and 12 weeks (or later) were also assessed.

The second type of experiment was designed to determine what happens to the LTC-ICs present in starting cell suspensions when these are plated directly in CFC assays. To answer this question, CFC assays were set up with ≈200 $CD34^+$ $CD38^-$ cells per ml and then all detectable colonies present after 14–19 days were individually plucked. The cells thus

Table 1. Demonstration of LTC-IC plating efficiency and self-renewal in assays of individual $CD34^+$ $CD38^-$ cells

Exp.	No. of single cells tested	No. of positive LTC-IC assays (%)		
		Week 6	Weeks 6 and 12	Week 12 only
1	180	19 (11)	7 (4)	18 (10)
2	60	7 (12)	1 (2)	4 (7)
	100	23 (23)	—	—
3	60	5 (8)	0 (0)	3 (5)
	120	27 (23)	—	—

Positive assays were those which contained ≥1 CFC when assayed at the times shown. Single cell cultures in experiment 1 were set up by manual plating; all others were set up by using the deposition unit of the flow cytometer. The percent of cultures that were positive after 6 weeks gives the frequency of LTC-ICs in the starting population. The proportion of these that also were positive after 12 weeks (last column) gives the frequency of self-renewing LTC-ICs (experiment 1, 7/19 = 37%; experiment 2, 1/7 = 14%; experiment 3, 0/5 = <20%).

obtained were pooled and washed, and aliquots were plated in new CFC and LTC-IC assay cultures. In addition, all of the cells present in the remaining methylcellulose in the cultures from which the colonies had been removed were separately harvested and then similarly pooled, washed, and assayed for CFCs and LTC-ICs. Most of the colonies present in the primary CFC assays appeared to be early-stage GM colonies that have the capacity, upon further incubation (for another 1–2 weeks) to achieve a very large size (14, 16). When the cells from these colonies were replated into secondary CFC assays, numerous colonies of multiple types were produced as expected (17). In contrast, in all but one experiment, aliquots of the same colonies did not contain LTC-ICs although substantial numbers of LTC-ICs (8–620% of input values) could be demonstrated in the background methylcellulose of the same primary CFC assays (Table 2). Interestingly, parallel CFC assays also showed the appearance of CFCs among the population present in the background methylcellulose of these 2- to 3-week-old primary CFC assays (data not shown).

Evidence of LTC-IC Amplification in Suspension Cultures in the Absence of Stroma. In a previous study, we showed that the same number of LTC-ICs could be maintained when highly purified starting populations of $CD34^+$ $HL\text{-}DR^{low}$ cells were cultured for 5 weeks under different conditions—i.e., in the presence of preestablished irradiated human marrow adherent layers or in their absence if exogenous SF, G-CSF, and IL-3 were added. Moreover, when both feeders and cytokines were present, LTC-IC recoveries remained the same as when either condition was used alone (18). This suggested that LTC-ICs can respond in a similar fashion to different growth factors, as was shown (19) for other types of primitive hematopoietic cells. Subsequently, we found that both LTC-IC expansion (7) and self-renewal (Table 1) could be obtained *in vitro.* We therefore hypothesized that some LTC-IC amplification might be demonstrable, at least at a clonal level, in medium containing a suitable growth factor mixture instead of stromal cells. Moreover, since extensive expansion of hematopoietic cells could be obtained from highly purified $CD34^+$ $CD45RA^-$ $CD71^-$ marrow cells (of which the $CD34^+$ $CD38^-$ cells used here represent a subset) in a completely defined (serum free) medium supplemented with various SF-containing cytokine mixtures (9), it seemed likely that the same serum substitute that supports such CFC expansion might also support LTC-IC amplification.

Table 2. LTC-IC maintenance in CFC assays of $CD34^+$ $CD38^-$ cells

CFC input no.	LTC-IC input no.	No. LTC-ICs recovered (%)	
		In colonies	In background MC
24	73	6 (0.8)	ND
<1	7	—	45 (620)
3*	20*	<1.5 (<0.8)*	3 (14)*
1†	20†	<1.5 (<0.8)†	17 (87)†
4	22	<2.5 (<1)	56 (250)
18	130	<0.8 (<0.1)	10 (8)

Progenitor numbers correspond to input values or yields from 200 input $CD34^+$ $CD38^-$ cells plated in primary methylcellulose (MC) cultures containing SF (50 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml), GM-CSF (20 ng/ml), and erythropoeitin (3 units/ml). In one case, cells were plated in MC assays to which Flt-3 ligand (50 ng/ml) was also added or not. All other values were derived from different marrow samples. LTC-IC values were calculated from their CFC output values on the assumption that for the LTC-IC and CFC assay conditions used here, 1 LTC-IC produces, on average, 10 CFCs detectable after 6 weeks (see Fig. 3). When no colonies were detected in the aliquot of cells assayed, the values shown are indicated as less than some number and represent the minimal number of progenitors (CFCs or LTC-ICs) that could have been detected given the size of the aliquot used for the determination. ND, not done. The mean number of CFCs per 200 starting cells was $<9 \pm 4$, and the mean number of LTC-ICs was 50 ± 22. The mean number of LTC-ICs recoverable from plucked colonies was $\leq 3 \pm 1$ (or $<1 \pm 0.2\%$ of the mean input number) and from the surrounding methylcellulose was 26 ± 10 (or $200 \pm 120\%$ of the mean input number).
*Cells in MC assay to which Flt-3 ligand at 50 ng/ml was not added.
†Cells in MC assay to which Flt-3 ligand at 50 ng/ml was added.

Accordingly, in two experiments, single $CD34^+$ $CD38^-$ cells were cultured in liquid serum-free cultures without feeders but in the presence of SF, Flt-3 ligand, IL-3, IL-6, G-CSF, and NGF, and then at intervals thereafter, each well was examined to determine whether a first cell division had occurred. Approximately one-third of the cells in both experiments appeared dead initially (or soon after plating) and occasionally no cell was ever seen. All of the other 240 cells proliferated within a 9-day period albeit after various periods of initial quiescence (Fig. 1). Thus at the end of first 9 days, clones ranging in size from 4 to 1000 cells (4–80 in one experiment and 5–1000 in the second experiment) had been produced. Each of these 240 clones was then harvested individually and the contents were assayed for LTC-ICs as shown in Table 3. For 160 of these clones, the harvested cells were divided equally between four replicate LTC-IC assays. For the other 80, two-thirds of each clone were plated into two replicate assays, but the remainder was transferred to fresh cytokine-supplemented serum-free medium. The latter were incubated at 37°C for a further 7 or 21 days and then all of the cells present finally were harvested and assayed well by well for the continuing presence of LTC-ICs (by plating the cells from each well in two replicate LTC-IC assay cultures). The two different periods of time used for the second phase of clonal expansion (7 and 21 days) were chosen to compensate for the different growth rates exhibited by the cells in the two experiments to try to keep the total number of cell divisions that would have occurred between the initiation and termination of the entire experiment as similar as possible in both cases. As shown in Table 3, 31% (37 of 118) and 52% (63 of 122) of all the 10-day-old clones in each of the two experiments contained ≥1 LTC-IC, 22% (26 of 118) and 28% (34 of 122) contained ≥2 LTC-ICs, and 4% (3 of 80) and 8% (6 of 80) contained at least 4 LTC-ICs. Analysis of the relative LTC-IC content of the 10-day-old clones (indicated by the proportion of replicate LTC-IC assays that were positive), as a function of the total number of cells in the clone at the time it was assessed, showed no obvious relationship (either positive or negative) between these two parameters (Fig. 2). However, it should be noted that no LTC-IC was found in any of the clones that contained >150 cells (a phenomenon that occurred only in the second experiment). Analysis of the LTC-IC content of the 80 reexpanded clones (after a total of 17 or 31 days) revealed LTC-ICs to be present in 23 clones, of which 4 (5%) had not contained a detectable LTC-IC in the aliquot tested after an initial 10 days

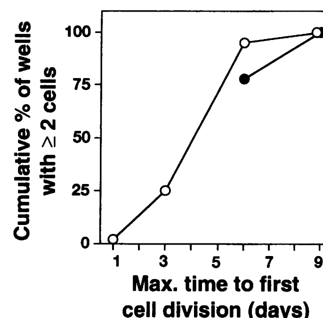

FIG. 1. Time course of initiation of proliferation of single viable $CD34^+$ $CD38^-$ cells cultured in serum-free medium containing SF (100 ng/ml), Flt-3 ligand (100 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml), and β-NGF (5 ng/ml). ●, Experiment 1; ○, experiment 2.

Table 3. LTC-IC content of clones produced from individual $CD34^+$ $CD38^-$ cells cultured for variable periods in serum-free medium containing SF, Flt-3 ligand, IL-3, IL-6, G-CSF, and NGF

Exp.	No. clones tested	Age of clones, days	% assayed for LTC-ICs	No. (%) of clones with *n* positive LTC-IC assay replicates for clones tested						No. (%) of reexpanded clones with LTC-ICs	
				$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n \geq 1$	$n \geq 2$	Maintained*	Further amplified†
1	80	10	100	19	11	11	3	44 (55)	25 (31)	—	—
	42	10	67	10	9	—	—	19 (45)	9 (21)	—	—
		31	33	11	6	—	—	17 (40)	6 (14)	13 (31)	4 (10)
2	80	10	100	8	10	5	6	29 (36)	21 (26)	—	—
	38	10	67	3	5	—	—	8 (21)	5 (13)	—	—
		17	33	3	3	—	—	6 (16)	3 (8)	6 (16)	0 (0)

*≥1 LTC-IC was present in the 67% of the cells in the 10-day-old primary clone that was assayed at that time for LTC-ICs and ≥1 LTC-IC was also detectable in the reexpanded clone.

†No LTC-IC was detected in the 67% of the cells in the 10-day-old primary clone that was assayed at that time for LTC-ICs but ≥2 LTC-ICs were detectable in the reexpanded clone.

of culture. In the 19 clones where LTC-ICs had been detectable after 10 days, (i.e., 24% of the 80 tested), LTC-ICs were still present 1–3 weeks later. In each of these experiments, the wells that did not appear to have been seeded initially with viable cells were also harvested and assayed for LTC-ICs at the end of the first 10 days of incubation; however, no LTC-IC was detected in these assays.

The results obtained from the replicate LTC-IC assays of the clonal progeny generated from single $CD34^+$ $CD38^-$ cells cultured for 10 days in stroma-free suspension cultures indicate that many of the clones contained multiple (≥4) LTC-ICs. To obtain a more precise estimate of the net change in LTC-IC numbers that occurred in these cultures over time, the total number of CFCs produced by all of the input LTC-ICs (assayed independently in standard LTC-IC assays of single cells from the same initial $CD34^+$ $CD38^-$ starting population) was compared to corresponding data from the LTC-IC assays of the 10-, 17-, and 31-day-old clones. These comparisons indicated a continuing overall LTC-IC expansion of up to almost 70-fold (Table 4). The validity of this method of calculating LTC-IC expansion is, however, critically dependent on the assumption that the LTC-IC produced *in vitro* are qualitatively similar to those present in the original $CD34^+$ $CD38^-$ fraction of normal marrow and, in particular, that their average CFC output remains unchanged. To examine this assumption, the distribution of CFC outputs from individual LTC-ICs in the $CD34^+$ $CD38^-$ starting population was compared with the distribution of CFC outputs from the LTC-IC in the 10-day-old clones where only one of the four replicates was positive (i.e., the probability that the positive assay had been initiated with >1 LTC-IC was <0.12). A comparison of these distributions for the two experiments performed is shown in Fig. 3. Individual LTC-ICs from both sources produced from 1 to ≈100 CFCs, and there was no evidence of any significant difference in their average CFC generating potential. Comparison of the relative numbers of different types of CFCs produced by the LTC-ICs in the input $CD34^+$ $CD38^-$ populations and those produced by the LTC-ICs present in the 10-day-old clones also revealed no obvious differences (ratios of granulocyte–macrophage colony-forming units to erythroid burst-forming units to granulocyte–erythroid–macrophage–megakaryocyte colony-forming units in both cases were ≈97:3:0.1).

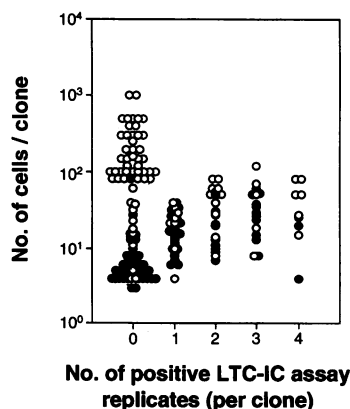

FIG. 2. Lack of relationship between the total number of cells produced within 9 days by single $CD34^+$ $CD38^-$ cells cultured as described in Fig. 1 and their content of LTC-ICs as evidenced by the number of replicate (up to four) LTC-IC assays performed on each that were positive. Symbols are as in Fig. 1.

DISCUSSION

In vivo data indicate that the proliferative potential of some hematopoietic stem cells is sufficient to be of major medical interest if it could be harnessed and exploited *in vitro*. To date, considerable progress has been made in the identification of cell sources and *in vitro* conditions that allow enormous numbers of mature cells and even their clonogenic precursors to be generated over periods of 2–4 weeks (8–10). However, such bursts of hematopoietic activity are not sustained and have not been associated with evidence of a significant net expansion of the stem cell pool, although accompanying short-term maintenance of such cells in the murine system has been achieved (6). Under culture conditions where less dramatic expansions of CFCs and terminal cells are obtained, some amplification of more primitive precursors, detectable as LTC-ICs, has been reported (7, 13). In the present study, we have shown that such amplifications may be due to the execution of LTC-IC self-renewal divisions. However, shortening of the latent period required for stromal cell-responsive CFC precursors to begin to generate progeny detectable as CFC in semisolid (methylcellulose-containing) assays also likely occurs. The extent of LTC-IC amplification we have now shown is achievable (≈50-fold in 2–4 weeks), and the fact that this can be obtained under defined culture conditions from a subpopulation of cells that can be readily and reproducibly

Table 4. Calculated overall LTC-IC amplification in cultures of single $CD34^+$ $CD38^-$ cells maintained for up to 3 weeks in serum-free medium supplemented with SF, Flt-3 ligand, IL-3, IL-6, G-CSF, and NGF

Exp.	Total no. of CFCs per 100 initial $CD34^+$ $CD38^-$ cells			Fold LTC-IC amplification	
	Input LTC-ICs	Day-10 clones	Day-17 or -31 clones	After 10 days	After 17–31 days
1	117	3940	7970	33	68 (31 days)
2	97	2750	4580	28	47 (17 days)

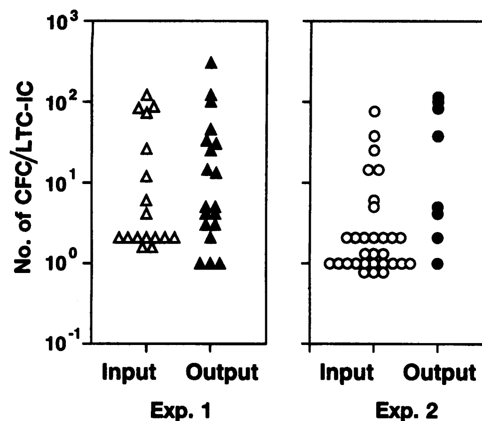

FIG. 3. Comparison of the distributions of CFC numbers produced by individual LTC-ICs in the $CD34^+$ $CD38^-$ fraction of normal marrow where assays of single cells were performed (input, open symbols) with CFC numbers produced in the LTC-IC assays of the 10-day-old clones (produced by $CD34^+$ $CD38^-$ cells) in serum-free cytokine-supplemented medium (output, solid symbols) for those clones where only one of four replicate assays was positive.

isolated from normal adult human marrow makes this procedure of immediate interest for a variety of biological and therapeutic applications. These include studies focused on an examination of the genetic requirements for preserving hematopoietic stem cell properties as well as clinical treatments requiring regeneration of the hematopoietic system with normal or genetically modified stem cells. It should, however, be noted that the extent of LTC-IC amplification achieved when higher concentrations of cells and/or less-purified marrow cells are cultured has been found to be much lower (ref. 7 and unpublished findings), indicating the sensitivity of this process to variations in the procedure followed.

At the present time, the molecular mechanisms that mediate the stromal cell-based stimulation of LTC-IC proliferation and differentiation are not known. Previous studies demonstrating the ability of immortalized murine fibroblasts to substitute for human marrow feeder layers in human LTC-IC assays (15, 20), including fibroblasts that are genetically unable to produce murine SF (18), have suggested that novel growth factors may be involved. More recently, the possibility that Flt-3 ligand may play a role in this regard has emerged (21–23). The cytokines used in the present studies were selected on the basis of previous (7) or concurrently (24) acquired data, indicating their ability to stimulate very primitive hematopoietic cell types of either human or murine origin. Subsequent experiments (to be described elsewhere) have shown that some of the cytokines included in the expansion medium used here were not necessary and that slightly higher LTC-IC amplifications can be achieved with higher concentrations of those cytokines that are important.

The present experiments also provide several other insights into previously unanticipated aspects of LTC-IC regulation. For example, they show that when $CD34^+$ $CD38^-$ cells are stimulated by soluble cytokines in stroma-free cultures to produce substantial numbers of daughter cells in a 10-day period (up to 1000), one-half to two-thirds of the original cells may not produce progeny within 10 days that are subsequently detectable as LTC-ICs, even though in some of these clones (4 of 80 = 5%; Table 3), LTC-IC can be detected after continued clonal amplification. Thus, initiation of cell division *per se* does not necessarily shorten the latent period required to detect all stromal cell-responsive CFC precursors. The present studies also confirm the finding that most human LTC-ICs can be stimulated to proliferate within 1.5 weeks in liquid culture, without loss of their ability to be detected as LTC-ICs. Interestingly, in the presence of methylcellulose, they are able to survive but not proliferate. Thus, at least within the $CD34^+$ $CD38^-$ fraction of normal marrow cells, LTC-ICs and CFCs appear as distinct and largely nonoverlapping progenitor types, although both are responsive to at least some of the same factors (24). Further experiments will clearly be required to establish the molecular mechanisms responsible for these differences.

We thank Gail Thornbury for assistance in operating the FACS, Irene Edelmann for secretarial assistance, StemCell for a generous gift of purified recombinant human erythropoietin, Amgen similarly for SF, Immunex for Flt-3 ligand, and Sandoz for IL-3 and GM-CSF. These studies were supported by a grant from the National Cancer Institute of Canada (NCIC) with funds from the Terry Fox Foundation. A.L.P. was supported by a grant (J01034-MED) from the E. Schrödinger Foundation and C.J.E. is a Terry Fox Cancer Research Scientist of the NCIC.

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