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Cytokine manipulation of primitive human hematopoietic cell self-renewal

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ABSTRACT **Previous studies have shown that primitive human hematopoietic cells detectable as long-term culture-initiating cells (LTC-ICs) and colony-forming cells (CFCs) can be amplified when $CD34^+$ $CD38^-$ marrow cells are cultured for 10 days in serum-free medium containing flt3 ligand (FL), Steel factor (SF), interleukin (IL)-3, IL-6, and granulocyte colony-stimulating factor. We now show that the generation of these two cell types in such cultures is differentially affected at the single cell level by changes in the concentrations of these cytokines. Thus, maximal expansion of LTC-ICs (60-fold) was obtained in the presence of 30 times more FL, SF, IL-3, IL-6, and granulocyte colony-stimulating factor than could concomitantly stimulate the near-maximal (280-fold) amplification of CFCs. Furthermore, the reduced ability of suboptimal cytokine concentrations to support the production of LTC-ICs could be ascribed to a differential response of the stimulated cells since this was not accompanied by a change in the number of input $CD34^+$ $CD38^-$ cells that proliferated. Reduced LTC-IC amplification in the absence of a significant effect on CFC generation also occurred when the concentrations of FL and SF were decreased but the concentration of IL-3 was high (as compared with cultures containing high levels of all three cytokines). To our knowledge, these findings provide the first evidence suggesting that extrinsically acting cytokines can alter the self-renewal behavior of primary human hematopoietic stem cells independent of effects on their viability or proliferation.**

In normal adult life, hematopoietic cell differentiation appears to span a large number of cell generations during the course of which marked changes occur both in the ability of the cells to respond to individual factors and in the biological consequences of those responses (1). Consequently, mechanisms regulating hematopoietic stem cell functions cannot be reliably inferred from studies of later cell types. The ability of a cell to generate and sustain the production of both mature myeloid and lymphoid cells for many weeks after being transplanted into a hematologically compromised host has been widely adopted as an operationally useful (albeit cumbersome) definition for its assignment to the stem cell compartment. In mice, cells with this property can be quantitated by appropriate limiting dilution analysis procedures (2). Transplantable human hematopoietic cells with lympho-myeloid reconstituting abilities have also been demonstrated (3, 4) and a method for their quantitation is under development (5). In the interim, much use has been made of surrogate measures of hematopoietic stem cell activities. Several lines of evidence indicate that cells referred to as long-term culture-initiating cells (LTC-ICs) are closely related to, if not highly overlapping with, transplantable stem cells (6). LTC-ICs are distinguished by their ability to generate myeloid colony-forming cells (CFCs) for at least 5 weeks in liquid cultures containing certain types of fibroblasts and can be quantitated by measurement of their CFC output at or after this time (6–9).

The recent development of methods for obtaining highly enriched populations of LTC-ICs and CFCs from normal human marrow (10–12) has facilitated the acquisition of considerable information about the responses of these cells to a wide variety of stimuli. For example, we have shown that the addition of a fourth cytokine [i.e., interleukin (IL)-6, granulocyte colony-stimulating factor (G-CSF), or nerve growth factor β] to a combination of flt3 ligand (FL), Steel factor (SF), and IL-3 enhances the production of CFCs (to values >500-fold above input numbers) in short-term (10 day) cultures of $CD34^+$ $CD38^-$ human marrow cells but does not further amplify the number of concomitantly produced LTC-ICs, which can increase 25-fold (13). Subsequent experiments suggested that the production of cells with the functional properties of LTC-ICs or CFCs might be differentially dependent on the concentration as well as the types of cytokines to which they were exposed (14). In addition, we found that LTC-ICs and CFCs in freshly isolated $CD34^+$ $CD38^-$ human marrow populations differ from CFC in their ability to proliferate in methylcellulose-containing medium (LTC-ICs being completely inhibited), even when stimulated by the same factors that stimulate LTC-IC expansion in liquid medium (12). The present studies were, therefore, undertaken to examine independently the cytokine concentration dependence of these four distinct responses of human marrow $CD34^+$ $CD38^-$ cells, i.e., LTC-IC amplification, CFC production, and cloning efficiency in liquid or semi-solid medium. The results obtained support a role for cytokines in influencing LTC-IC self-renewal decisions.

MATERIALS AND METHODS

Purification and Analysis of Bone Marrow Cells. Light-density cells (<1.077 g/cm^3) were isolated from previously frozen cadaveric human bone marrow (Northwest Tissue Centre, Seattle, WA) using Ficoll/Paque (Pharmacia). Cells to be further purified were first washed in PBS containing 2% fetal calf serum (FCS, from StemCell Technologies, Vancouver, BC), resuspended in Hanks' Hepes-buffered salt solution containing 2% FCS and 0.1% sodium azide (HFN) and then incubated simultaneously at $\leq 10^7$ cells per ml for 30 min at 4°C

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Abbreviations: LTC-IC, long-term culture-initiating cell; CFC, colony-forming cell; FL, flt3 ligand; SF, Steel factor; IL, interleukin; G-CSF, granulocyte colony-stimulating factor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; PE, phycoerythrin; CFU, colony-forming unit(s); PI, propidium iodide.

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with monoclonal antibodies specific for CD34 (8G12-Cy5), CD45RA [8d2-R-phycoerythrin (PE)], CD71 [OKT9-fluorescein isothiocyanate (FITC)], or CD34 (8G12-FITC) and CD38 (Leu17-PE). Cells were then washed once in HFN and once in HFN containing propidium iodide (PI; p-5264, Sigma; 2 μg/ml) prior to resuspension in Hanks' Hepes-buffered salt solution containing 2% FCS for isolation of $CD34^+$ $CD38^-$, $CD34^+$ $CD45RA^+$ $CD71^-$, or $CD34^+$ $CD45RA^-$ $CD71^+$ populations. In the experiments where the phenotype of cultured cells was to be investigated, anti-CD45RA-FITC and anti-CD33 (D3HL60.251)-FITC (Immunotech, Westbrook, ME) were used in addition to anti-CD34 (8G12)-Cy5, anti-CD71-FITC, and anti-CD38-PE for the isolation of $CD34^+$ $CD38^-$ $CD45RA^-$ $CD71^-$ $CD33^-$ cells. For analyses of cultured cells, the cells were washed as described above and then stained with anti-CD34 (8G12)-Cy5, anti-CD38-PE, and one of the following, anti-CD45RA-FITC, anti-CD71-FITC, or anti-CD33-FITC. For the single cell cloning experiments, recombinant human AnnexinV-FITC (Bio-Whittaker) was used in addition to anti-CD34 (8G12)-Cy5 and anti-CD38-PE for the isolation of $AnnexinV^-$ $CD34^+$ $CD38^-$ cells. Cells were sorted on a FACStar Plus (Becton Dickinson) equipped with a 5-W argon laser and a 30-mW helium laser. Viable (PI^-) cells with low–medium forward scattering and low side scattering characteristics and the desired surface antigen phenotype were collected in Eppendorf tubes containing serum-free Iscove's medium. Cells regarded as positive were those showing greater fluorescence than 99.9% of all cells stained with a matched fluorochrome-labeled irrelevant isotype control antibody. A more detailed description of all of the reagents and procedures used has been published (9, 11, 12).

Stromal-Free Liquid Suspension Cultures. Sorted cells were cultured at 37°C for 10 days in Iscove's medium containing a defined serum substitute (BIT, StemCell Technologies), low density lipoproteins (Sigma; 40 μg/ml), and 5×10^{-5} M 2-mercaptoethanol (Sigma) at 1 or 200 cells per 100 μl per well in 96-well plates as described (12), with purified recombinant human IL-3 (Sandoz Pharmaceutical), G-CSF (Amgen Biologicals), SF (Amgen Biologicals), IL-6 (Cangene, Mississauga, ON), FL (Immunex, Seattle), and/or nerve growth factor β (R & D Systems) added as indicated. For single cell cultures, individual cells were deposited by the sorter into wells preloaded with the desired medium and the wells were then examined 1–2 days later to identify those that contained a single viable (refractile) cell. After 10 days, clone size determinations were made by counting the number of viable cells in each well.

Factorial Design Analysis Experiments. A 2^3 orthogonal factorial analysis (15) was used to investigate the dependence of LTC-IC expansion on the relative concentration of FL, SF, and IL-3 in the medium. These cytokines were tested at 10 and 300 ng/ml for SF and FL and 2 and 60 ng/ml for IL-3. Each of the eight cytokine combinations possible was tested with three sources of $CD34^+$ $CD38^-$ cells. In addition, each separate experiment included four replicate cultures containing FL, SF, and IL-3 at 60, 60, and 10 ng/ml, respectively. The logarithm of the LTC-IC expansion measured (see below) with each mixture was then normalized for each separate marrow by subtracting the mean of all the logarithmic LTC-IC expansions and dividing the resultant value by the standard deviation of all the logarithmic LTC-IC expansions. This transformation allowed the results from the three marrows to be analyzed as a single data set using the JASS 2.0 software program (Joiner Associates, Madison, WI) (13).

Progenitor Assays. Cell suspensions were assayed for erythropoietic [erythroid colony-forming units (CFU-E) and erythroid burst-forming units (BFU-E)], granulopoietic [granulocyte–macrophage CFU (CFU-GM)], and multilineage (CFU-GEMM) CFCs in Iscove's medium containing 0.9% methylcellulose, 30% FCS, 1% bovine serum albumin, and 10^{-4} M 2-mercaptoethanol (Methocult H4430; StemCell Technologies), supplemented with erythropoietin (StemCell Technologies), SF, IL-3, IL-6, G-CSF, and GM-CSF (Sandoz Pharmaceuticals) as described (9, 13). LTC-IC numbers were generally determined in bulk assays (or, as indicated in selected experiments, by limit dilution analysis) using irradiated genetically engineered murine fibroblast feeders producing human G-CSF, IL-3, and SF, also as described (7, 9). These engineered feeders increase the number of LTC-ICs detectable in normal marrow (using a 6-week CFC production endpoint) by a factor of ~10 and also increase the average output of CFCs per LTC-IC from 7 to 18 (9).

Statistical Analysis. Statistical significance of differences between test populations were determined by either one or two-tailed Student's *t* tests.

RESULTS

Differential Cytokine Dose Dependence of LTC-IC and CFC Expansion. In a first series of experiments, aliquots of $CD34^+$ $CD38^-$ marrow cells were incubated in serum-free medium supplemented with various concentrations of a cytokine mixture [FL/SF/IL-3/IL-6/G-CSF, 5:5:1:1:1 (wt/wt)]. After 10 days of culture, the numbers of LTC-ICs, CFCs, and total nucleated cells were determined and compared with corresponding input values for that experiment. The results pooled from seven such experiments are shown in Fig. 1. In these, input values were 8.2 ± 3.3 LTC-ICs and 7.3 ± 2.6 CFCs per 100 input $CD34^+$ $CD38^-$ cells. Maximum expansion of LTC-IC numbers (62 ± 22-fold) required stimulation of the cells by FL (300 ng/ml), SF (300 ng/ml), IL-3 (60 ng/ml), IL-6 (60 ng/ml), and G-CSF (60 ng/ml). In contrast, near maximally increased CFC numbers (280-fold) were obtained with 30-fold lower concentrations of the same cytokine mixture.

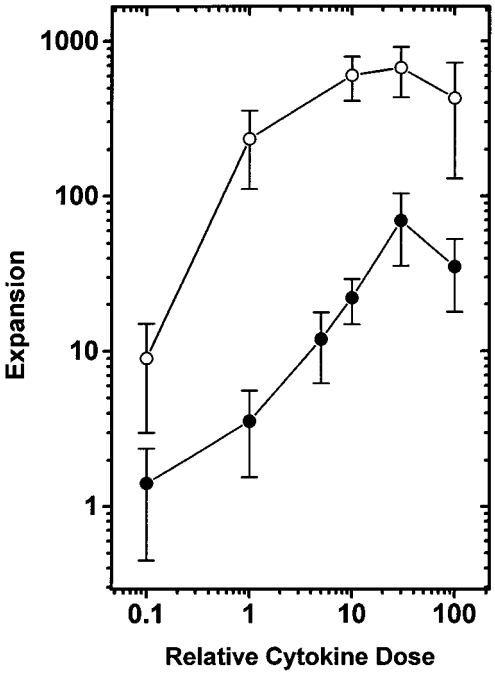

FIG. 1. Expansion of LTC-IC (●) and CFC (○) numbers (relative to input) in 10-day 100-μl serum-free cultures initiated with 200 $CD34^+$ $CD38^-$ cells. Input LTC-IC and CFC numbers were 8.2 ± 3.3 and 7.3 ± 2.6 per 100 $CD34^+$ $CD38^-$ cells, respectively. A relative cytokine dose of 1 represents SF at 10 ng/ml, FL at 10 ng/ml, IL-3 at 2 ng/ml, IL-6 at 2 ng/ml, and G-CSF at 2 ng/ml. Points represent the mean ± SEM of data from three to seven experiments performed with three to five bone marrow samples.

Interestingly, the total number of cells produced increased progressively as the concentration of the cytokine mixture added was increased with the greatest expansion (78 ± 9-fold more than input) being obtained in the cultures containing the highest concentration of cytokines tested (FL at 1 μg/ml, SF at 1 μg/ml, IL-3 at 200 ng/ml, IL-6 at 200 ng/ml, and G-CSF at 200 ng/ml). As a result, although ≈15% of the initial $CD34^+$ $CD38^-$ cells were either CFC or LTC-IC, after 10 days in FL at 10–100 ng/ml, SF at 10–100 ng/ml, IL-3 at 2–20 ng/ml, IL-6 at 2–20 ng/ml, and G-CSF at 2–20 ng/ml, all of the cells present were detectable as CFCs. For example, in one experiment 100 input $CD34^+$ $CD38^-$ cells generated ~4500 cells, 4400 ± 300 CFCs and 200 ± 30 LTC-ICs after 10 days in FL at 100 ng/ml, SF at 100 ng/ml, IL-3 at 20 ng/ml, IL-6 at 20 ng/ml, and G-CSF at 20 ng/ml. Thus, some of the cultured cells would most likely have had the capacity to be detected both as CFCs and LTC-ICs.

The magnitude of the LTC-IC expansions calculated for cultures supplemented with FL at 300 ng/ml, SF at 300 ng/ml, IL-3 at 30 ng/ml, and IL-6 at 30 ng/ml (Fig. 1) were based on measurements of CFC output values from "bulk" LTC-IC assays. To determine whether the LTC-ICs generated in these cultures had the same proliferative potential as the input LTC-IC, their average CFC outputs (at 6 weeks) were compared. These were obtained from LTC-IC assays in which the test cells were seeded at concentrations that produced <22 ± 6% positive cultures (i.e., <4 ± 2% of these would have been seeded with >1 LTC-IC, $P = 0.05$). The resultant average CFCs per LTC-IC values proved to be the same: 19 ± 27 CFCs per LTC-IC (mean ± SD; range, 1–94) for the cultured LTC-ICs vs. 14 ± 24 (range, 1–108) for the LTC-ICs in the starting population of $CD34^+$ $CD38^-$ marrow cells. These values are also the same as that previously reported for the LTC-IC population in unseparated normal marrow (see *Materials and Methods*).

As noted previously (13, 16), a readily detectable proportion of the input LTC-IC population (11 ± 4%) was able to survive for a period of 10 days in the absence of any added cytokines, and even quite low levels of these cytokines (FL at 1 ng/ml, SF at 1 ng/ml, IL-3 at 0.2 ng/ml, IL-6 at 0.2 ng/ml, and G-CSF at 0.2 ng/ml) were sufficient to maintain LTC-IC numbers at input levels for 10 days (1.5 ± 1.2-fold expansion more than input). When the concentration of the five-cytokine mixture used in these experiments was increased above FL at 300 ng/ml, SF at 300 ng/ml, IL-3 at 60 ng/ml, IL-6 at 60 ng/ml, and G-CSF at 60 ng/ml, the yield of LTC-ICs and CFCs decreased slightly, in spite of the fact that there was a further increase in the total number of cells produced.

The marked difference in the cytokine dose dependence of LTC-IC and CFC production revealed in these experiments could be due to differences in the viability or mitogenic responsiveness of different subsets of $CD34^+$ $CD38^-$ cells or, alternatively, to a difference in the cytokine-stimulation requirement for the same cells to maintain or acquire the functions necessary for detection as LTC-ICs or CFCs. To discriminate between these alternatives, the frequency and size of clones generated by single PI^- $AnnexinV^-$ $CD34^+$ $CD38^-$ cells cultured in the presence of either (*i*) FL at 300 ng/ml, SF at 300 ng/ml, IL-3 at 60 ng/ml, IL-6 at 60 ng/ml, and G-CSF at 60 ng/ml or (*ii*) a 10-fold lower concentration of the same cytokine combination were compared in two experiments. The results obtained in both were similar. As can be seen in the example shown in Fig. 2*A*, the yield of clones was similar for the two culture conditions compared ($P = 0.4$), in spite of the fact that the production of LTC-ICs was again selectively reduced (≈50-fold) with only a minimal decrease in CFC production (≈25%) in the cultures maintained at the lower cytokine concentration. (In these experiments the LTC-IC expansions were 57 ± 18- vs. 1.2 ± 0.5-fold, $P < 0.1$, in the high vs. low cytokine concentration mixtures, respectively, with corresponding CFC expansions of 570 ± 50- and 430 ± 90-fold, $P = 0.5$).

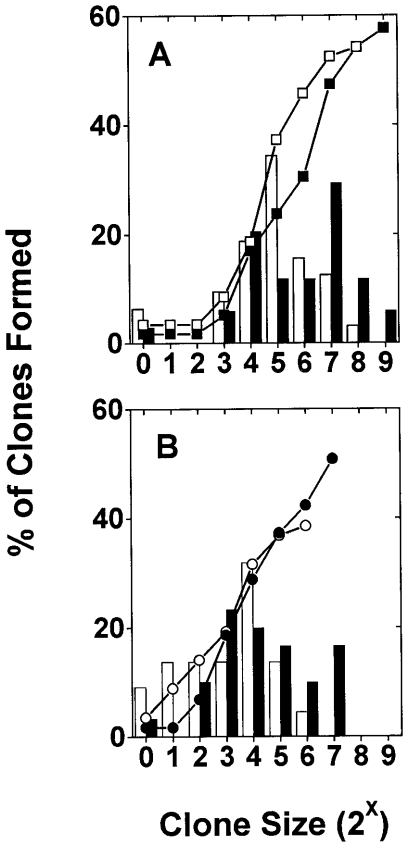

FIG. 2. Size distribution (bars) and cumulative frequencies (symbols) of clones generated from a representative experiment in which single PI^- $AnnexinV^-$ $CD34^+$ $CD38^-$ cells were cultured for 10 days in serum-free medium supplemented with (*i*) FL at 300 ng/ml, SF at 300 ng/ml, IL-3 at 60 ng/ml, IL-6 at 60 ng/ml, and G-CSF at 60 ng/ml (solid bars and ■, $n = 59$ wells) or (*ii*) FL at 30 ng/ml, SF at 30 ng/ml, IL-3 at 6 ng/ml, IL-6 at 6 ng/ml, and G-CSF at 6 ng/ml (open bars and □, $n = 59$ wells) (*A*) or (*iii*) FL at 300 ng/ml, SF at 300 ng/ml, and IL-3 at 60 ng/ml (solid bars, ●, $n = 59$ wells) or (*iv*) FL at 10 ng/ml, SF at 10 ng/ml, and IL-3 at 60 ng/ml (open bars and ○, $n = 57$ wells) (*B*). At the end of the 10 days, all cells from all clones generated under a given set of conditions were pooled and assayed for their content of CFCs and LTC-ICs. The results of these assays confirmed the results shown in Fig. 1 (for the comparison undertaken in *A*) and in Table 1 (for the comparison undertaken in *B*) (see *Results*).

Cytokine Concentration Dependence of Colony Formation by Different Subpopulations of CFCs in Methylcellulose Cultures. Given the difference seen in the cytokine concentration requirements for LTC-IC and CFC expansion by $CD34^+$ $CD38^-$ marrow cells in liquid suspension cultures (Fig. 1), as well as previous evidence that the freshly isolated LTC-ICs are not detectable as CFCs in methylcellulose (12), we were interested in determining the cytokine concentration dependence of colony formation (in methylcellulose) by $CD34^+$ $CD38^-$ marrow cells. However, because the majority of CFCs in the marrow are not found within the $CD34^+$ $CD38^-$ fraction, these studies were extended to include an examination of the CFCs in other fractions. Therefore, $CD34^+$ $CD45RA^+$ $CD71^-$ cells (in which all of the CFCs are granulopoietic) and $CD34^+$ $CD45RA^-$ $CD71^+$ cells (in which most of the CFCs are erythroid) (10) were also isolated and cultured in methylcellulose containing the same range of concentrations of the five-factor mixture used in the experiments described in Fig. 1. From all three cell fractions, maximum or close to maximum numbers of most types of colonies were generated in all cultures independent of the concentration of

cytokines added over a 300-fold range starting at FL at 1 ng/ml, SF at 1 ng/ml, IL-3 at 0.2 ng/ml, IL-6 at 0.2 ng/ml, and G-CSF at 0.2 ng/ml (Fig. 3). The only major exceptions to this were the granulopoietic CFCs and the multilineage CFCs present in the $CD34^+$ $CD45RA^-$ $CD71^+$ fraction, which required higher cytokine concentrations to be optimally stimulated. Peak CFC plating efficiencies (measured in cultures containing FL at 100 ng/ml, SF at 100 ng/ml, IL-3 at 20 ng/ml, IL-6 at 20 ng/ml, and G-CSF at 20 ng/ml) were 7 ± 4%, 14 ± 5%, and 22 ± 6% for the $CD38^-$, $CD45RA^+$ $CD71^-$, and $CD45RA^-$ $CD71^+$ subpopulations of $CD34^+$ cells, respectively. In the assays of the latter two subpopulations, colony formation by the more mature types of CFCs that they contain (identified by a reduced proliferative potential) was also favored at the lower cytokine concentrations (data not shown).

Analysis of the Effect of Independently Varying the Concentrations of FL, SF, and IL-3 on LTC-IC and CFC Expansion. We next investigated the potential effects of independently altering the concentrations of individual cytokines that stimulate LTC-IC expansion (relative to input values) in 10-day liquid suspension cultures of $CD34^+$ $CD38^-$ cells. For this, a 2^3 factorial design analysis was used. The combined results of the three experiments performed are shown in Table 1. As anticipated from the dose–response studies shown in Fig. 1, the greatest expansion of LTC-ICs occurred when all three cytokines were present at the higher of the two concentrations tested (FL at 300 ng/ml, SF at 300 ng/ml, and IL-3 at 60 ng/ml). Analysis of the normalized effect of each combination of different cytokine concentrations tested showed that, for the three cytokines considered (FL, SF, and IL-3), LTC-IC expansion was primarily and significantly ($P < 0.05$) dependent on the presence of a high concentration of FL. Thus high numbers of LTC-ICs could be obtained when the concentration(s) of SF, IL-3, or both were reduced, as long as the concentration of FL was kept high. Conversely, when the concentration of FL was low, elevating the concentration of only SF, or even both SF and IL-3, did not significantly enhance LTC-IC expansion over that observed when all three factors were present at a low level. Interestingly, when only the concentration of IL-3 was elevated, the yield of LTC-ICs was reduced even further ($P < 0.1$, compared directly by the one-tailed Student's t test, $n = 3$, to that observed when all three cytokines were present at the lower levels). This markedly negative effect of a relatively high concentration of IL-3 was, however, not observed when a high concentration of either FL or SF was present. Of note, CFC expansion in the same experiments (Table 1) was not significantly affected by the presence of an elevated concentration of FL ($P = 0.4$), although an elevated level of IL-3 did consistently allow significantly more ($P < 0.01$) CFCs to be produced, in keeping with the possibility that exposure of LTC-ICs to high concentrations of IL-3 may, under certain circumstances, promote their differentiation.

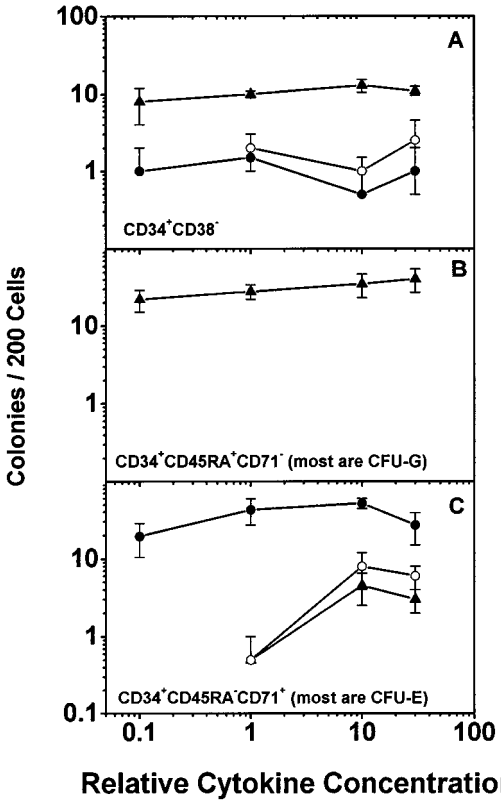

FIG. 3. Number of colonies generated from 200 $CD34^+$ $CD38^-$ (*A*), $CD34^+$ $CD45RA^+$ $CD71^-$ (*B*), or $CD34^+$ $CD45RA^-$ $CD71^+$ cells (*C*) plated directly in methylcellulose containing different concentrations of the same mixture of five cytokines used in the experiments shown in Fig. 1. Points for erythroid (●, from CFU-E and BFU-E), mixed (○, from CFU-GEMM), and myeloid (▲, from CFU-GM) colonies represent the mean ± SEM of values obtained from four experiments.

To determine whether the negative effect of high concentrations of IL-3 on the production of LTC-ICs in 10-day suspension cultures of $CD34^+$ $CD38^-$ cells was associated with a failure of a subset of these to remain viable or to divide, additional experiments with single cell cultures were performed. In these, individual PI^- $AnnexinV^-$ $CD34^+$ $CD38^-$ cells were cultured for 10 days in either the optimal (for LTC-IC expansion; i.e., FL at 300 ng/ml, SF at 300 ng/ml, and IL-3 at 60 ng/ml) or the suppressive (FL at 10 ng/ml, SF at 10 ng/ml, and IL-3 at 60 ng/ml) mixture. At the end of this time, the number of clones and their sizes were determined by visual inspection. The results of two such experiments (one of which is shown in Fig. 2*B*), indicated that the mixture favoring LTC-IC expansion stimulated the formation of slightly more ($P < 0.10$) and larger ($P < 0.01$) clones than the mixture that suppressed LTC-ICs. This suggests that an excess of IL-3 can be directly toxic for some $CD34^+$ $CD38^-$ cells that have the potential to generate LTC-IC progeny. Measurements of the number of LTC-ICs and CFCs produced in parallel bulk cultures showed the same effects as previously obtained in the factorial design experiments (i.e., LTC-IC expansions of 10.5 ± 0.6- vs. 1.1 ± 0.4-fold, $P < 0.05$, and CFC expansions of 420 ± 210- vs. 230 ± 100-fold, $P = 0.12$, in the high IL-3, SF, and FL vs. the high IL-3 plus low SF and FL mixtures, respectively). The proportion of $CD34^+$ $CD38^-$ cells that formed clones was also significantly higher ($P < 0.05$) in cultures containing FL at 300 ng/ml, SF at 300 ng/ml, and IL-3 at 60 ng/ml when G-CSF at 60 ng/ml and IL-6 at 60 ng/ml were also present. In addition, for each pair of conditions assessed, the average size of the clones was always higher when the cytokine concentration was higher ($P < 0.01$).

Characterization of the Phenotypes of the Cytokine-Expanded Populations. Fig. 4 shows the surface antigen profiles of the cells generated in 10-day serum-free cultures in the presence of either (*i*) FL at 100 ng/ml, SF at 100 ng/ml, IL-3 at 20 ng/ml, IL-6 at 20 ng/ml, and G-CSF at 20 ng/ml or (*ii*) FL at 300 ng/ml, SF at 300 ng/ml, and IL-3 at 60 ng/ml. The first of these mixtures was chosen because it had been shown to support maximal expansion of CFCs and near-maximal expansion of LTC-ICs (ref. 13 and Fig. 1). The second mixture was chosen because it was found to support maximal expansion of LTC-ICs and significant expansion of CFCs (Table 1). Cultures were initiated with $CD34^+$ $CD38^-$ $CD45RA^-$ $CD71^-$ $CD33^-$ cells and the cells present after 10 days were then analyzed for their surface expression of CD34, CD38, CD33, CD45RA, and CD71. After 10 days, the total cell number in the cultures containing the five-cytokine combination had increased 74 ± 19-fold and 42 ± 11-fold in cultures

Table 1. Differential effects of altering the relative concentrations of FL, SF, and IL-3 on LTC-IC and CFC expansion

Cytokine concentration, ng/ml			Progenitor expansion*		Analysis of effect on LTC-IC expansion	
FL	SF	IL-3	LTC-IC	CFC	Normalized effect†	Significance of normalized effect‡
300	300	60	45 ± 15	76 ± 19	−0.29	NS
300	300	2	33 ± 12	31 ± 5	0.85	NS
300	10	60	22 ± 6	76 ± 28	−0.26	NS
300	10	2	18 ± 9	41 ± 30	1.2	$P < 0.05$
10	300	60	4 ± 2	62 ± 33	0.73	NS
10	300	2	4 ± 3	39 ± 11	0.49	NS
10	10	2	4 ± 1	28 ± 15	N/A	N/A
10	10	60	0.3 ± 0.1	68 ± 37	−0.83	$P < 0.10$

*Values shown are the mean ± SEM of data from three experiments.
†Effects of individual cytokines (main effects) within the mixture were calculated from the sum of differences between all of the normalized LTC-IC expansion values (see *Materials and Methods*) obtained with that cytokine at the higher vs. the lower concentration tested [the levels of the other cytokines being constant (15)]. Effects of elevated levels of multiple cytokines (interaction effects) were determined by analysis of variance (15). All effects were determined relative to the cultures where all three cytokines were present at the lower level such that a change in cytokine concentration with no overall effect would have a value of 0. The SEM of the normalized effect on LTC-IC expansion was 0.24.
‡Significance was determined from a comparison of the normalized effect and the SEM of the normalized effect of the elevated cytokine concentrations on LTC-IC expansion. NS, not significant (i.e., $P > 0.1$); N/A, not applicable.

containing the three-cytokine mixture ($n = 3$). Of these, 85 ± 16% and 62 ± 15%, respectively, were $CD34^+$. Within these $CD34^+$ cell populations, 45 ± 23% (five cytokines) and 88 ± 9% (three cytokines) of the cells were still $CD38^-$. Thus, the total number of $CD34^+$ $CD38^-$ cells present in the five- and three-factor supplemented cultures increased by ≈21- and ≈32-fold, respectively. Interestingly, very few of these expanded $CD34^+$ $CD38^-$ cells (<1% for the five-factor combination and ≈6% for the three-factor combination) were still $CD33^-$, $CD71^-$, or $CD45RA^-$. In fact, most of the $CD34^+$ cells present after 10 days were positive for all of these antigens.

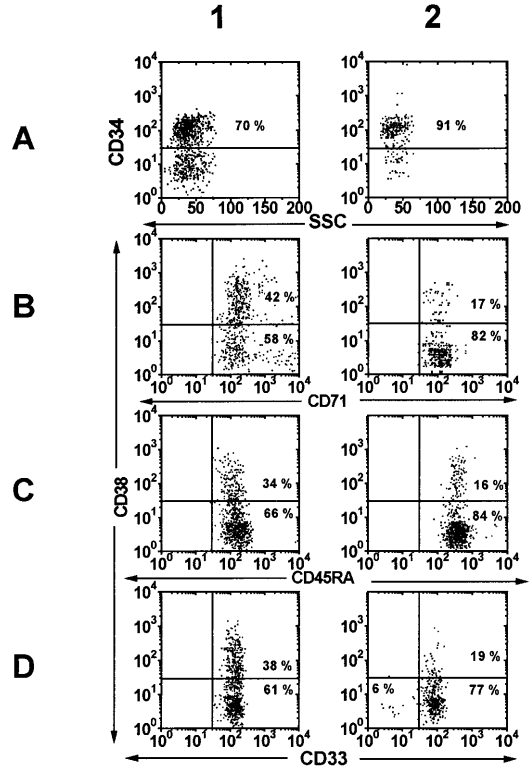

FIG. 4. Representative flow cytometric analyses of CD34 expression vs. side scatter (SSC) (row A) and within the $CD34^+$ cell population for expression of CD38 and CD71 (row B), CD45RA (row C), or CD33 (row D) on subpopulations of viable (PI^-) cells generated from 200 input $CD34^+$ $CD38^-$ Lin^- cells after 10 days in serum-free cultures supplemented with either FL at 100 ng/ml, SF at 100 ng/ml, IL-3 at 20 ng/ml, IL-6 at 20 ng/ml, and G-CSF at 20 ng/ml (column 1) or FL at 300 ng/ml, SF at 300 ng/ml, and IL-3 at 60 ng/ml (column 2). The lower left-hand quadrant in each plot represents boundaries set by 99.9% of the unstained and isotype-labeled antibody controls.

DISCUSSION

Four important and novel findings are reported herein. The first is to document a higher rate of LTC-IC amplification (≥50-fold in 10 days) in cultures of $CD34^+$ $CD38^-$ human marrow cells than has been previously described. Moreover, the LTC-ICs thus generated display the same average, albeit highly variable, proliferative behavior as the LTC-ICs in the original $CD34^+$ $CD38^-$ marrow population. Interestingly, the *in vitro*-amplified cells expressed phenotypes that are not expressed by normal marrow cells (11, 17, 18). In addition, some of the amplified LTC-ICs must have acquired an ability to proliferate in semi-solid medium (since under certain conditions, all of the cultured cells could be detected as CFC), a situation that contrasts with the LTC-ICs present in the original marrow cell suspensions (12). These findings indicate that the combination of attributes associated with very primitive cells generated during normal hematopoiesis *in vivo* are not necessarily coordinately regulated, as noted for primitive murine hematopoietic cells expanding either *in vitro* (19) or *in vivo* (20). The possible biological consequences of a primitive hematopoietic cell undergoing some phenotypic changes normally associated with its differentiation while, at the same time, retaining certain functional properties indicative of its original state await further investigation.

The second major finding reported herein is the demonstrated importance of the *relative* concentration of the cytokines in a given mixture to support LTC-IC amplification in serum-free cultures of $CD34^+$ $CD38^-$ marrow cells. In particular, the present studies show that this response is most sensitive to the concentration of FL present in a combination of cytokines that consists of FL, SF, and IL-3. This is reminiscent of our previous finding that, of a large number of cytokines tested including SF and IL-3, FL is on its own the most potent stimulator of LTC-IC expansion (13).

The third observation of note is that high levels of IL-3 can have a direct and negative impact on LTC-IC expansion when both FL and SF levels are relatively low and that this negative effect can be overcome if either the FL or SF concentration is sufficiently increased. An ability of IL-3 to block the cytokine-stimulated generation of lymphoid precursors from primitive murine hematopoietic cells with lympho-myeloid potential and to impair the self-renewal of the latter *in vitro* has recently been

reported by Ogawa and coworkers (21, 22). The present demonstration of a similar action of IL-3 on human LTC-IC amplification suggests the likelihood of a parallelism in signal transducing events in analogous types of IL-3-stimulated populations of primitive murine and human hematopoietic cells. The present observations also underscore the possibility of detrimental consequences of including IL-3 in cytokine mixtures used for the activation or expansion of stem cell transplants in clinical protocols. On the other hand, although Ogawa and coworkers (23) also identified factors that could override the negative effects of IL-3 on murine cells, these did not include either FL or SF, whereas we found both of these factors to have this salvaging activity on primitive human cells (LTC-ICs). This latter discrepancy, with our finding that human CFC expansion is enhanced in combinations containing a high concentration of IL-3 (some of which can reduce or suppress human LTC-IC expansion), suggests the possibility of some interesting differences between the two species.

The fourth point of interest is the evidence of a unique cytokine concentration dependence of LTC-IC amplification from $CD34^+$ $CD38^-$ cells. This dose effect was found to be independent of the ability of the input cells to remain viable, proliferate, and generate CFCs, as none of these latter responses were significantly affected when the cytokine concentration was decreased to the point that LTC-IC expansion was reduced 25- to 50-fold. We have shown (12) that in the presence of a high cytokine concentration of the cytokines tested, >30% of all the input $CD34^+$ $CD38^-$ cells that are viable under these conditions generate progeny LTC-ICs. The present findings suggest that all of the 10-day progeny can be CFCs. These findings argue that the retention or loss of LTC-IC potential by the progeny of freshly isolated $CD34^+$ $CD38^-$ marrow cells is influenced by the concentration of cytokines to which the cells are exposed. Thus, the different cytokine dose–effect relationships characterizing LTC-IC and CFC expansion (Fig. 1) would appear to reflect the differential cytokine concentration requirements for these two outcomes from a common precursor population rather than the requirements of two distinct types of $CD34^+$ $CD38^-$ cells characterized by different cytokine sensitivities [as has been shown for other classes of marrow progenitors (24)]. Another example of the same cells responding differently to different concentrations of cytokines was reported by Dexter *et al.* (25). They showed that IL-3-dependent murine FDCP-mix cells can proliferate in the presence of lower concentrations of IL-3 than are necessary for concomitant maintenance of their pluripotent state. Our findings, like those of Dexter *et al.* (25), are thus supportive of a model of cytokine-induced signaling in which the biological response may be modified not only by the type(s) of factors to which the cell is exposed but also by the duration of such interactions and the number of receptors bound per unit time (26). To our knowledge, the present studies provide the first evidence that this model is relevant to the control of primary human hematopoietic stem cell self-renewal. They thus focus interest on the identification of differences in the intracellular signaling pathways that must be able to direct (or block) primitive hematopoietic cell differentiation when these cells are exposed to cytokine mixtures that elicit different biological outcomes.

The extent of human LTC-IC expansion shown herein to be possible also has important practical implications in terms of clinical stem cell transplants. The ability to achieve ≥50-fold expansions of LTC-ICs and, at the same time, >500-fold amplifications of CFCs, reinforces the future potential of *in vitro* technology to manipulate these cells for a variety of clinical applications. It is now clear that an important consideration in the design of any *in vitro* procedure or bioreactor system will be the provision of a mechanism that can maintain high extracellular levels of stem-cell-stimulatory cytokines and also remove factors that may be selectively inhibitory. Interestingly, we have recently shown that the very hematopoietic cell types whose maximum proliferation *in vitro* depends on their stimulation by the highest concentration(s) of cytokines also exhibit the greatest capacity to deplete the same cytokines from the medium (14). Thus, these findings may explain why the identification of conditions that support the expansion of hematopoietic stem cells has been so elusive.

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