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Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers

(long-term marrow culture)

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ABSTRACT **A major goal of current hematopoiesis research is to develop *in vitro* methods suitable for the measurement and characterization of stem cells with long-term *in vivo* repopulating potential. Previous studies from several centers have suggested the presence in normal human or murine marrow of a population of very primitive cells that are biologically, physically, and pharmacologically different from cells detectable by short-term colony assays and that can give rise to the latter in long-term cultures (LTCs) containing a competent stromal cell layer. In this report, we show that such cultures can be used to provide a quantitative assay for human "LTC-initiating cells" based on an assessment of the number of clonogenic cells present after 5–8 weeks. Production of derivative clonogenic cells is shown to be absolutely dependent on the presence of a stromal cell feeder. When this requirement is met, the clonogenic cell output (determined by assessment of 5-week-old cultures) is linearly related to the input cell number over a wide range of cell concentrations. Using limiting dilution analysis techniques, we have established the frequency of LTC-initiating cells in normal human marrow to be ≈1 per 2 × 10^4 cells and in a highly purified CD34-positive subpopulation to be ≈1 per 50–100 cells. The proliferative capacity exhibited by individual LTC-initiating cells cultured under apparently identical culture conditions was found to be highly variable. Values for the number of clonogenic cells per LTC-initiating cell in 5-week-old cultures ranged from 1 to 30 (the average being 4) with similar levels being detected in positive 8-week-old cultures. Some LTC-initiating cells are multipotent as evidenced by their generation of erythroid as well as granulopoietic progeny. The availability of a system for quantitative analysis of the proliferative and differentiative behavior of this newly defined compartment of primitive human hematopoietic cells should facilitate future studies of specific genetic or microenvironmental parameters involved in the regulation of these cells.**

Several lines of evidence suggest that mouse marrow contains a hierarchy of primitive cells distinguishable from one another by their differing capacities for sustaining hematopoiesis after transplantation into lethally irradiated or genetically defective recipients (1–4). The most primitive of these cells are defined by their superior long-term reconstituting ability in competitive transplantation assays (4, 5) and by their capacity for generating lymphoid as well as myeloid progeny (6–10). Such cells are physically separable from the majority of cells detectable by short-term *in vivo* or *in vitro* clonogenic assays, indicating that they represent a distinct population (2, 4).

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Less is known about human hematopoietic stem cells, although available data suggest a close parallelism with the murine system. For example, recent studies of circulating blood cells in patients transplanted with marrow from normal female donors heterozygous for certain restriction fragment length polymorphisms at the X chromosome-linked *PGK* or *HPRT* loci have provided evidence for clonal granulocytes and T cells originating from a common, donor-derived precursor, thus indicating the presence of transplantable lymphomyeloid stem cells in normal adult human marrow (11). Furthermore, *in vitro* exposure of human marrow cells to agents such as 4-hydroperoxycyclophosphamide (4-HC) at doses that kill ≈90% of cells detectable by *in vitro* colony assays (12) has been found to spare the ability of the same marrow to serve as a protective autograft (13), again suggesting little overlap between human clonogenic progenitors and reconstituting cells.

In searching for an *in vitro* assay to allow the ultimate purification and functional characterization of the most primitive stem cell populations in human marrow, we have focused attention on the long-term marrow culture (LTC) system (14). When such cultures are initiated with unseparated mouse or human marrow cells, granulocytes and macrophages are continuously produced for several months. This is accompanied by the continuous turnover (15) and differentiation of nonadherent clonogenic granulopoietic cells whose numbers are, in turn, sustained by their continuous release from the adherent layer of the culture (16). Interestingly, for human LTCs it has been shown that the hematopoietic cells in the original marrow that give rise to the myeloid progenitor cells detectable 4–8 weeks later are much less sensitive to 4-HC than are directly clonogenic cells (17).

In LTCs initiated with murine marrow, lymphomyeloid reconstituting cells are known to be maintained for at least 4 weeks (18–21), suggesting the potential suitability of analogous human cultures to support the maintenance and measurement of human hematopoietic stem cells with similar properties. As yet it has not been possible to test this prediction directly, as such studies with human cells are necessarily limited to experiments that can be performed *in vitro* and conditions that support expression of the lymphopoietic potential of the most primitive hematopoietic cells in human marrow have not been identified (22). Nevertheless, some progress has recently been made in characterizing human cells that express myelopoietic potential in LTC (23, 24). In particular, we have recently shown that the number of

Abbreviations: BFU-E, burst-forming unit, erythroid; CFU-GM, colony-forming unit, granulocyte–macrophage; CFU-GEMM, colony-forming unit, granulocyte erythroid–megakaryocyte macrophage; 4-HC, 4-hydroperoxycyclophosphamide; LTC, long-term culture; FLS, forward light scatter.

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clonogenic myeloid progenitors present after 5 weeks defines a population of primitive human "LTC-initiating cells" that can be readily enriched several hundredfold (23) and at the same time are physically separated from the majority (>95%) of the clonogenic progenitors present in the original marrow sample. These experiments did not, however, provide absolute values for the content of LTC-initiating cells in the suspensions tested, nor did they allow an assessment to be made of the proliferative and differentiative potentialities expressed by individual LTC-initiating cells maintained in the LTC system—i.e., in the presence of semiconfluent, irradiated human marrow adherent (stromal) cell layers. In the present study, we have successfully used the LTC system with limiting numbers of input cells to allow investigation of each of these parameters.

METHODS

Cells. Heparinized marrow was obtained from informed and consenting individuals donating marrow for allogeneic transplantation. Low-density cells (<1.068 g/ml) were isolated on a discontinuous Percoll density gradient (23), resuspended in Iscove's medium with 50% fetal calf serum, and kept overnight at 4°C. Nucleated cell recovery after density centrifugation was 15% ± 2%, clonogenic cell recovery was 95% ± 18%, and the recovery of LTC-initiating cells as indicated by the number of clonogenic progenitors detectable after 5 weeks in LTC was 93% ± 29%. The overall progenitor enrichment over buffy coat values was ≈7-fold.

Cell Purification. Low-density cells were first incubated with 10 μg of purified anti-CD34 per ml (anti-My10; generously provided by C. Civin, Johns Hopkins University, Baltimore) and then stained with fluorescein isothiocyanate-conjugated $F(ab)_2$ fragments of sheep anti-mouse IgG (Organon Teknika). Cells to be sorted for My10 expression alone were suspended with 2 μg of propidium iodide per ml. Cells to be double-stained were washed and stained with anti-HLA-DR conjugated directly to phycoerythrin (Becton Dickinson).

Cells were analyzed and sorted on a FACS 440 or a FACStar^{PLUS} (BD FACS Systems, Becton Dickinson). In either case, the sorter was calibrated prior to each run with 10-μm fluorescent microspheres and compensation was set up for double-stained specimens in both fluorescein isothiocyanate and phycoerythrin fluorescence with single-stained specimens. All cells were sorted within a low orthogonal light scatter window (23). The forward light scatter (FLS) sort window was set to include all nucleated cells in some cases, or to include only cells in the lymphocyte region (FLS^{low}), specifically excluding the blast and monocytic region, in other cases, as specified in the text. Cells were also sorted for either high My10 expression (My10^{++}) and low propidium iodide binding or high My10 expression and low or negative HLA-DR expression (HLA-DR^{low}) as described (23). Cells were sorted in the full drop envelope mode on the FACS 440 and in the counter mode on the FACStar^{Plus} at 2000–3000 cells per sec and collected in Iscove's medium with 50% fetal calf serum.

LTCs. Cultures were established and maintained in general according to standard procedures (23, 25) with appropriate scaling down of the number of cells used and the volume of medium added each week according to the surface area of the culture dish or well. Buffy coat cells were seeded at 10^6 cells per cm^2, low density cells at 10^3–10^5 cells per cm^2, and sorted cells at lower concentrations (30–10^3 cells per cm^2) depending on the enrichment of LTC-initiating cells anticipated. For limiting dilution experiments, LTCs were established in 6-mm wells in 96-well flat-bottomed Nunclon microwell plates (Nunc) with 100 μl of LTC medium per well, and these were seeded with 500–10^4 low-density cells per well or 10–400 of the sorted cells per well. In these analyses, each cell suspension was seeded at three or four different initial cell concentrations with a mean of 23 ± 1 replicate wells per concentration. Except where specified, all LTCs were initiated by seeding the test cells on semiconfluent irradiated (15 Gy of 250-kilovolt peak x-rays) human marrow feeder layers subcultured from previously established LTC adherent layers (23). After 5 or 8 weeks, the nonadherent cells and the adherent cells suspended by treatment with trypsin (25) were washed and plated in standard methylcellulose assays (25) to determine the total clonogenic cell content of each LTC [i.e., the number of primitive erythropoietic (burst-forming unit, erythroid; BFU-E), granulopoietic (colony-forming unit, granulocyte–macrophage; CFU-GM), and multilineage (colony-forming unit, granulocyte erythroid–megakaryocyte macrophage; CFU-GEMM) progenitors].

RESULTS

Stromal Feeder Requirement of Human LTC-Initiating Cells. In murine LTCs, the maintenance of cells that are clonogenic either *in vitro* or *in vivo* has been shown to depend on a mechanism involving direct cell–cell interactions between primitive hematopoietic cells and ontogenetically unrelated stromal cells (26, 27). Together, these populations constitute a significant proportion of the adherent layer of LTCs (27, 28). An adherent layer composed of a mixture of similar cell types also forms in LTCs initiated with human marrow (14, 29) and the most primitive hematopoietic cells are also found almost exclusively within this fraction (25). Nevertheless, it has been difficult to investigate the importance of the stromal cell component of LTCs initiated with human marrow because of problems in obtaining functional test populations sufficiently depleted of stromal cells and their precursors.

In seven separate experiments, we evaluated the presumed requirement for a functional stromal feeder by comparing the 5-week clonogenic progenitor content of cultures initiated by seeding up to 7000 cells per dish from a sorted (My10^{++}, HLA-DR^{low}, FLS^{low}) population of low-density human marrow cells [highly enriched for LTC-initiating cells (23)] into dishes with or without preestablished feeder layers of irradiated adherent marrow cells. In culture dishes without feeders, no visible adherent layer formed. This was consistently associated with a marked (although not total) and significant ($P < 0.01$; Student's t test) reduction in the number of clonogenic progeny detectable in 5-week-old cultures (mean value ± 1 SEM = 1.1% ± 0.5% of values in control cultures with feeders).

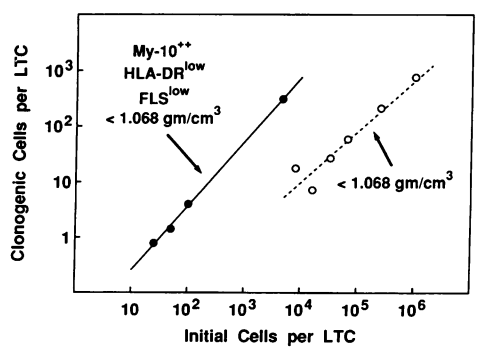

FIG. 1. The number of clonogenic cells per LTC at 5 weeks is plotted against the number of initial cells seeded into the LTC for a representative experiment with cells isolated on a Percoll density gradient (○; slope of the log of the values = 0.91) and a representative experiment with light density, My10^{++}, HLA-DR^{low}, FLS^{low} cells (●; slope of the log of the values = 1.02).

Table 1. Linearity of clonogenic progenitor numbers after 5 weeks in LTC as a function of the number of cells seeded per LTC

Cells	No. of experiments	Mean slope ± SEM	Probability slope = 1
Percoll gradient (unsorted)	11	0.88 ± 0.05	$P > 0.05$
$MY10^{++}$	2	0.91 ± 0.06	$P > 0.2$
$MY10^{++}$, $HLA\text{-}DR^{low}$	2	1.00 ± 0.12	$P > 0.9$
$MY10^{++}$, $HLA\text{-}DR^{low}$, FLS^{low}	7	1.57 ± 0.39	$P > 0.1$

P values were derived from a Student's *t* test, which tested the null hypothesis that the mean slope observed was not significantly different from 1.0.

Clonogenic Progenitor Output Is Linearly Related to the Number of Marrow Cells Assayed. We next examined the relationship between the number of cells placed into a LTC and the number of clonogenic progenitors present 5 weeks later, both for low-density marrow cell suspensions and for various subpopulations of $My10^{++}$ cells, which, on a per cell basis, yield at least 100 times more clonogenic progenitors after 5 weeks in culture on supportive feeders. The mean number of clonogenic progenitors per LTC at 5 weeks was determined for each concentration at which cells were initially added, and the results were then used to calculate the slope of the logarithm of the input/output values. Two examples are shown diagrammatically in Fig. 1. The pooled data for all experiments performed with each type of cell suspension, in which at least three cell concentrations were assessed in any given experiment, are shown in Table 1. In no case was the mean slope value found to differ significantly from 1.0 ($P > 0.05$; Student's *t* test). Thus, the number of clonogenic progenitors detectable after 5 weeks is linearly related to the number of cells assayed over a wide range of input cell concentrations. Moreover, this holds true regardless of the presence or absence of a variety of mature cell types that are present in the low-density fraction of normal marrow and that are removed by the sorting procedure used to enrich for LTC-initiating cells.

Quantitation of LTC-Initiating Cells by Limiting Dilution Analysis. Although the number of clonogenic cells present after 5 weeks provides a quantitative and hence useful measure of the LTC-initiating cell frequency in the original population, only relative values are obtained. To obtain an absolute measure of these cells, mini-LTCs were established in 96-well plates containing preestablished irradiated adherent layer cells. For each evaluation at least three cell concentrations were used with 20–24 replicates per concentration. The frequency of negative wells (no clonogenic progenitors detectable 5 weeks later) was then determined and the frequency of LTC-initiating cells in the starting population

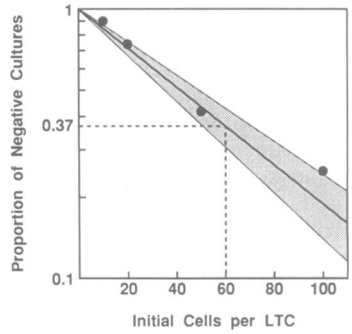

FIG. 2. Limiting dilution analysis of data from a representative experiment in which decreasing numbers of light-density, $My10^{++}$, $HLA\text{-}DR^{low}$, FLS^{low} cells were seeded onto irradiated marrow feeders and the number of clonogenic cells detectable after 5 weeks was then determined. In this experiment, the frequency of LTC-initiating cells in the starting cell suspension (i.e., the reciprocal of the concentration of test cells that gave 37% negative cultures) was 1 per 60 cells or 1.7% of all nucleated cells initially present.

was calculated by Poisson statistics and the weighted mean method (30, 31) with iterative procedures to determine the best linear fit and standard errors of this function (Fig. 2). Since the Percoll density separation step gives an ≈7-fold enrichment in LTC-initiating cells over buffy coat cell suspensions, the frequency of LTC-initiating cells in unseparated bone marrow could be calculated and was found to be ≈1 per 2×10^4 cells. Using a four-parameter FACS sorting procedure to select cells expressing a high level of My10 (CD34), a low or undetectable level of HLA-DR, and showing low orthogonal light scatter properties, we were able to isolate a population in which the frequency of LTC-initiating cells was 1–2% (Table 2). This represents an overall enrichment of 200- to 400-fold (by comparison to normal marrow buffy coat). There was no significant difference between the enrichment of LTC-initiating cells in the $My10^{++}$, $HLA\text{-}DR^{low}$ cell fractions with or without gating only the FLS^{low} cells ($P > 0.1$; Student's *t* test). This did, however, consistently eliminate a proportion of directly clonogenic progenitors, although on average, the frequency of clonogenic cells (4.1%) was 3 times higher than that of LTC-initiating cells (1.3%) in the $My10^{++}$, $HLA\text{-}DR^{low}$, FLS^{low} fraction. Nevertheless, in one experiment, the frequency of LTC-initiating cells (13 per 1000) did exceed the frequency of directly clonogenic cells (7 per 1000).

In a few experiments, duplicate sets of LTCs were used to analyze the frequency of cells capable of producing clonogenic progenitors detectable at 8 as well as 5 weeks. Using the 8-week endpoint, the frequency of LTC-initiating cells was, on average, ≈2-fold lower than that obtained using the 5-week endpoint (Table 2).

Proliferative Properties of LTC-Initiating Cells. To investigate the proliferative potential of LTC-initiating cells, we

Table 2. Absolute frequencies of LTC-initiating cells

Cells	No. of experiments	% recovery*		LTC-initiating cell frequency†	
		Nucleated cells	LTC-initiating cells	5 weeks	8 weeks‡
Percoll gradient	5	100	100	0.037 ± 0.002	0.015 ± 0.005 ($n = 2$)
$MY10^{++}$	3	3.9 ± 0.4	76 ± 13	0.58 ± 0.21	
$My10^{++}$, $HLA\text{-}DR^{low}$	3	0.7 ± 0.2	29 ± 12	2.3 ± 0.9	0.5 ($n = 1$)
$MY10^{++}$, $HLA\text{-}DR^{low}$, FLS^{low}	7	0.8 ± 0.1	55 ± 19	1.3 ± 0.1	1.2 ± 0.8 ($n = 2$)

*Mean ± SEM expressed as percent of values in Percoll gradient marrow cell suspensions.
†Frequency per 100 nucleated cells in the population tested (mean ± SEM).
‡In a subset of *n* experiments, duplicate dishes were evaluated after 8 weeks in LTC.

Table 3. Proliferative potential of LTC-initiating cells

Cells	Progenitors per LTC-initiating cell*	
	5 weeks	8 weeks
Percoll gradient	4.6 ± 0.9	5.7 ± 1.3
$MY10^{++}$	3.7 ± 0.9	
$MY10^{++}$, $HLA\text{-}DR^{low}$	4.4 ± 1.0	3.8
$MY10^{++}$, $HLA\text{-}DR^{low}$, FLS^{low}	4.2 ± 0.5	3.1 ± 2.0

*Calculated by multiplying the frequency of LTC-initiating cells in each experiment (determined by limiting dilution assays) by the total number of cells plated in all LTCs to determine the total number of LTC-initiating cells for that experiment. The total content of clonogenic progenitors in all LTCs for an individual experiment was obtained directly from clonogenic progenitor assays. Numbers of experiments from which each mean value (±SEM) was derived are the same as in Table 2.

determined both the average and the range of clonogenic cell numbers in individual 5-week-old LTCs initiated by limiting numbers of cells. The average number of clonogenic progenitors present at the 5-week time point was 4 and this value remained the same regardless of the purity of the population initially added ($P > 0.1$; analysis of variance) (Table 3). Moreover, from several experiments in which duplicate cultures were set up, it was found that the number of clonogenic progenitors per LTC-initiating cell still averaged 4.2 ± 1.0 after 8 weeks, a value not significantly different ($P > 0.1$; analysis of variance) from that obtained for 5-week-old cultures.

The range in clonogenic cell output values for individual LTC-initiating cells (assessed after 5 weeks of culture) was then determined by analyzing data for only those cultures in which the initial concentration of LTC-initiating cells (as determined by data from the entire experiment) was ≤0.31 per culture. At this concentration, the likelihood that all of the clonogenic progenitors detected in any given positive culture had derived from a single LTC-initiating cell is >85% (32). The resultant frequency distribution is shown in Fig. 3. These data were then used to derive a theoretical frequency distribution of clonogenic progeny numbers expected from individual LTC-initiating cells by subtracting 15% of a derived two LTC-initiating cell distribution from the experimental data (see Fig. 3 legend). The final derived distribution for single LTC-initiating cells and the data for ≤0.31 LTC-initiating cells per culture are, nevertheless, very similar. From the former, it appears that some LTC-initiating cells are capable of producing up to at least 30 clonogenic progenitors.

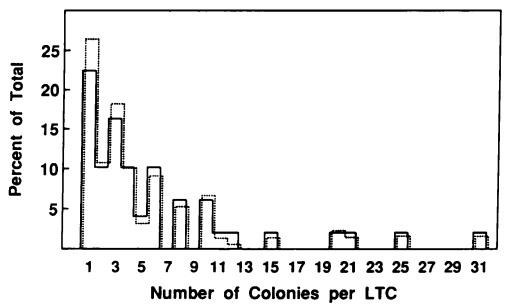

FIG. 3. Frequency distributions of the number of clonogenic progenitors detected after 5 weeks in a total of 189 LTCs each set up by seeding a limiting number of LTC-initiating cells (≤0.31 per LTC) onto preestablished feeders. Positive LTCs ($n = 47$) are those in which at least 1 clonogenic cell was detected. Experimental data (solid line) are compared to a theoretical frequency distribution of clonogenic progenitors from single LTC-initiating cells (dotted line) derived from the data as follows: Positive cultures set up with ≤0.31 LTC-initiating cells per culture have a ≤15% chance of having been seeded with >1 LTC-initiating cell (32). On the first iteration, we assumed that all the observed cultures were derived from a single LTC-initiating cell. We then calculated the expected distribution of clonogenic cell production from two LTC-initiating cells per culture by tabulating all pairwise combinations of the single-cell distribution (multiplying their observed frequencies and adding their clonogenic cell production). An adjusted single-cell distribution was then calculated by subtracting for each class in the observed distribution 15% of the calculated two-cell distribution for the class. This adjusted single-cell distribution was then used to calculate a new two-cell distribution, which was then used to calculate a second adjusted single-cell distribution. The dotted line represents the adjusted single-cell distribution after 10 such iterations.

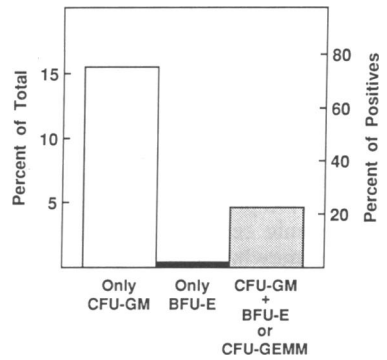

FIG. 4. Comparison of the number of 5-week-old LTCs plated with ≤0.31 LTC-initiating cells per culture that were found to contain only CFU-GM, only BFU-E, or a combination of CFU-GM and BFU-E or CFU-GEMM. (No other combinations were observed.) Data are from the same LTCs analyzed in Fig. 3 for total clonogenic cell content. Using a two-way test of independence, the probability that a CFU-GM and a BFU-E or CFU-GEMM occurred together at the frequency observed by chance alone is $P < 0.001$.

Differentiative Properties of LTC-Initiating Cells. Data from the same LTCs initiated with ≤0.31 LTC-initiating cells per culture were also analyzed for the type of clonogenic progenitors present after 5 weeks. In the majority of cultures, these were exclusively of the granulocyte–macrophage lineage (Fig. 4). However, in ≈20% of positive cultures, some progenitors with erythropoietic or multilineage potential were seen (i.e., BFU-E and/or CFU-GEMM). Almost all of these cultures also contained some CFU-GM and this association was not independent ($P < 0.001$; two-way test of independence), indicating that at least some LTC-initiating cells have the capacity to generate progeny that differentiate along different lineages.

DISCUSSION

In this study, we have described a quantitative assay for a very primitive hematopoietic cell in human marrow that is identified on the basis of its ability to give rise to progeny clonogenic cells in the presence of irradiated stromal feeder layers also of human marrow origin. The assay is linear over a wide range of input cell numbers both for unseparated marrow as well as for highly purified cell suspensions. This validates the applicability of this assay to the measurement of LTC-initiating cell numbers in a variety of experimental and clinical situations and hence opens up new opportunities for investigating both intrinsic (genetic) and extrinsic (microenvironmental) parameters that may influence the regulation of very primitive human hematopoietic cells *in vitro* and *in vivo*.

As a first application of this approach, we have used limiting dilution analysis techniques to quantitate the concentration of these LTC-initiating cells in normal human marrow and in various purified subpopulations assessed by

using a 5-week clonogenic cell output endpoint. Their frequency in unseparated marrow is ≈1 per 2×10^4 cells—i.e., ≈30-fold less than the frequency of clonogenic cells (CFU-GM plus BFU-E plus CFU-GEMM) and comparable to frequencies of cells that generate "blast" cell colonies, although reported values for the latter vary widely depending on assay conditions (33, 34). The use of an 8-week rather than a 5-week culture period preceding assessment of the number of daughter clonogenic cells produced detects a LTC-initiating cell that is somewhat less frequent in normal human marrow. Interestingly, this latter type of LTC-initiating cell (detected by using the 8-week endpoint) also appears to be more resistant to 4-HC (17), suggesting that it is more primitive. However, whether it is a truly distinct cell type or represents a subpopulation of the LTC-initiating cells identified by using the 5-week endpoint cannot be determined from available data since both are copurified in the most enriched populations currently obtainable (23).

Assessment of the number and type of clonogenic cells present in cultures seeded with limiting numbers of LTC-initiating cells (i.e., as low as 10 cells per well from the most highly purified populations) has also provided information about their proliferative and differentiative capacities. From analysis of a large number of such cultures, the proliferative capacity of individual LTC-initiating cells was found to vary widely even when maintained under the same conditions and assessed at the same time, with some LTC-initiating cells generating up to 30 clonogenic cells detectable after 5 weeks. A similar variability in the proliferative potential exhibited by individual pluripotent clonogenic cells during colony formation in semisolid medium has been documented (35), and it has been suggested that this may reflect the operation of a probabilistic mechanism contributing to the regulation of stem cell decisions to undergo terminal differentiation (36). The average number of clonogenic progenitors per LTC-initiating cell assessed after 5 weeks was found to be 4 and the same average value was also obtained for positive 8-week-old cultures. This proliferative function is clearly dependent on the presence of the cells in the irradiated stromal feeder, since highly purified $My10^{++}$ $HLA\text{-}DR^{low}$ cells in the low FLS and low to medium orthogonal light scatter (lymphocyte) window fail to produce clonogenic cells in the absence of a feeder (and also do not themselves contain stromal cells or their precursors).

Although the majority of clonogenic progenitors produced in the presence of a competent adherent layer appear to be restricted to the generation of granulocytes or macrophages, ≈20% of the LTC-initiating cells could also be shown to generate cells with erythropoietic potential. This may represent the true fraction of LTC-initiating cells that are multipotent or an underestimation. Measurements of the type and number of clonogenic cell output are limited by the same reliance on a single time point to evaluate the progeny produced from any given LTC-initiating cell. In addition, it is not known whether the culture conditions used here to detect LTC-initiating cells are optimal for the generation of clonogenic progeny. Indeed, this seems unlikely given the intermittent pattern of primitive progenitor proliferative activity previously shown to occur in these cultures (37) and the identification of strategies to alter this pattern (38).

The ability to distinguish and hence separate clonogenic cells from a more primitive population from which they derive should now make it possible to identify environmental conditions that may influence the earliest steps in human hematopoietic cell development. For example, altered self-renewal of LTC-initiating cells versus altered output of clonogenic cell progeny can now be separately assessed and quantitated. In the future, such information should serve as a useful starting point for investigating the molecular basis of how these processes may be uncoupled in various hematological malignancies (39).

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