Direct Comparison by Limiting Dilution Analysis of Long-Term Culture-Initiating Cells in Human Bone Marrow, Umbilical Cord Blood, and Blood Stem Cells

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Limiting-dilution analysis of long-term culture-initiating cells (LTCIC) is a quantitative method of estimating hematopoietic stem cell activity in clinical samples. We compared the numbers of LTCIC in bone marrow (BM), umbilical cord blood, and blood progenitor cells (obtained from patients with solid tumors at leukapheresis after mobilization with induction chemotherapy and filgrastim administration), using a two-stage long-term culture system and a limiting-dilution technique, scoring cobblestone areas of greater than 15 hematopoietic cells weekly for up to 8 weeks. Samples were obtained from 30 normal BMs, 20 human umbilical cords, and 32 leukapheresis products. Direct comparison of LTCIC in the three sources showed that the median proportions of cells generating hematopoietic foci from unfractonated mononuclear cells at 5 and 8 weeks, respectively, were 1:13,314 and 1:33,949 for BM, 1:12,506 and 1:34,546 for umbilical cord blood, and 1:10,302 and 1:12,891 for leukapheresis product. The estimated proportions of LTCIC from unfractonated mononuclear cells and CD34+ cells were similar in experiments with leukapheresis product. Leukapheresis product was superior to umbilical cord blood and cord blood to BM at 5 and 8 weeks of culture (P = .01). In two-stage long-term cultures, more colonies per flask and CD34+ cells were found in assays of leukapheresis product than in BM or umbilical cord blood cultures (P = .0005). Results obtained by this simplified limiting-dilution analysis correlated well with standard long-term cultures and can be used as a measure of the stem cell population. These data suggest that the incidence of putative stem cells in leukapheresis product and umbilical cord blood are at least comparable with that of BM.

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cense technique was used to label cells with a fluorochrome-conju-
gated anti–HPCA-2 CD34 monoclonal antibody (Becton-Dickinson,
Meylan, France). The percentage of CD34+ cells was estimated on
a fluorescence activated cell sorterr (FACS) using forward angle light
scatter and gating all live cells.3 CD34+ cells were separated on a
Ceprate (Cell-Pro Inc, Bothell, WA) immunoaffinity column using
the manufacturer’s protocol or sorted by FACS.

CFC assays. Ficoll-separated mononuclear cells were plated in
IMDM supplemented with 4 × 10^{-6} M/L glutamine, 10^{-7} M/L
sodium selenite, 2.5 × 10^{-4} M/L α-thioglycollate, 30% FCS, 10% 
medium conditioned by the bladder carcinoma cell line 5637 (as a
source of growth factors), 1% deionized bovine serum albumin
(Sigma, St Louis, MO), and 2 U of recombinant erythropoietin (Ci-
lag, High Wycombe, UK). Triplicate cultures were set up with 10^7
mononuclear cells/mL in standard 24-well tissue culture plates (Fal-
con; Becton-Dickinson). The plates were incubated at 37°C in
5% CO2, and 5%

Macrophage (GM-CFC), total erythroid (BFU-E), early erythroid
(BFU-E), late erythroid (BFU-EI), or multipotent (Mix-CFC) pro-
genitor cells, according to established criteria.10

Long-term cultures. The capacity of the mononuclear cells to
repopulate BM stroma was tested in two-stage long-term cultures.10
BM stromal cells for these cultures were obtained from normal
allogeic transplant donors. Stromal layers from the same donor
were used to test all samples from each patient. After 4 to 8 weeks
of culture, these stromal cultures were exposed to 16 Gy γ irradiation
from a 137Cesium source at a rate of 6.28 cGy/s to ablate endogenous
hematopoiesis. The growth medium was removed, leaving irradiated
stromal layers ready for inoculation of the test hemopoietic cells.

Ficoll-separated and adherent-cell—depleted BM and peripheral
blood mononuclear cells were inoculated on preformed irradiated
stromal layers. Adherent cells were removed by overnight incubation
in plastic flasks containing

Y

meth-

CO2,

5%
mol/L glutamine, mol/L deionized bovine serum albumin

Y

20 to 30 replicates in the informative range were required to
give a reliable estimate for dilutions using a binomial model.12

Some experiments were terminated at week 5 and cloning CFC
assays performed on each replicate well. The adherent layer was
washed and treated with 25% trypsin at 37°C. After 10 minutes,
trypsinsation was stopped by adding fetal calf serum. After centrifug-
ation for 10 minutes at 400g, the adherent cells were resuspended
in their corresponding supernatant and a standard GM-CFC assay
in agar performed.19

Statistical methods. Comparisons were made using standard sta-
tistical tests. Results are expressed as median ± range except where
otherwise stated. Baseline comparisons used Kruskal-Wallis tests.
Long-term culture profiles were compared using repeated measures
analyses of variance, assuming missing data to be missing at random.
An unstructured covariance matrix was assumed and reported tests
on differences between means are likelihood ratio tests. Estimates of
LTICIC proportions were analyzed by standard limiting-dilution
assay techniques.12 The assumptions of this model are: (1) Homoge-
neous suspension of LTICIC in each dilution. (2) A single LTICIC
in a well will result in a positive cobblestone area, ie, a single-hit
model.

RESULTS

Samples were obtained from 30 normal BM donors, 20
umbilical cords, and 32 leukapheresis procedures. The num-
ber of CFCs and the proportion of CD34+ cells in samples
containing 10^7 mononuclear cells from the freshly collected
normal BM, cord blood, and leukapheresis product were com-
pared. GM-CFC numbers were significantly higher in the
leukapheresis harvest (median, 4,255, range, 834 to
5

9,200) than in normal BM (median, 1,170, range, 300 to
3,740) or umbilical cord blood (median, 1,825, range, 520
to 3,400; P = .0005, Kruskal-Wallis). In addition, the pro-
portion of CD34+ cells was significantly higher in the leu-
kapoiesis product (median, 3.7%, range, 0.5 to 18) than in
normal BM (median, 1.48%, range, 0.4 to 8.3) or umbilical
cord blood (median, 1.13%, range, 0.56 to 3.9; P = .002,
Kruskal-Wallis).

Standard long-term cultures. The behavior of hemato-
poietic cells from BM, umbilical cord blood, and leukap-
hesis product in standard long-term cultures are compared in
Fig 2. Although in some cultures the numbers of supernatant
CFCs increased between weeks 0 and 5, by week 8 there
were fewer than week 9 in every case. The slope (rate of
decay) in mean numbers of supernatant CFC did not differ
significantly between the three groups (P = .11). However,
numbers of supernatant CFC per inoculum follow a Poisson
distribution (P < .0001). The leukapheresis product gave rise to significantly
more supernatant CFC than umbilical cord blood (P = .001)
and umbilical cord blood CFC counts were higher than that
of BM (P = .08). These data show a higher capacity to
generate CFC in the leukapheresis products than in normal
BM or umbilical cord blood but give no indication of the
number of stem cells.

Estimation of LTICIC in leukapheresis product by limiting-
dilution analysis. Data from two representative assays are
shown in Fig 3 on a complementary log-log plot. Assuming
that n follows a binomial distribution, and that the number
of LTICIC in any inoculum follows a Poisson distribution,
the following linear relationship holds:
LTCIC IN MARROW, CORD BLOOD, AND BLOOD STEM CELLS

Fig 1. Phase-contrast micrograph of a small cobblestone area at week 5 of culture. Mononuclear cells were inoculated on irradiated, preformed allogeneic stroma at week 1. The phase dark cells are cobblestone areas.

\[ \ln \left[ -\ln (1 - P_j) \right] = \ln (n_j) + \ln \phi \]

where, at the jth dilution step: \( r_j \) is the number of positive wells; \( n_j \) is the number of wells inoculated; \( P_j \) is the fixed probability of well being positive; \( x_j \) is the number of cells in the inoculum; and \( \phi \) is the proportion of LTCIC in the inoculum to be estimated.

This equation describes a straight line with slope 1.0 and intercept \( \ln \phi \) that was fitted using a maximum likelihood estimate with the statistical modeling package generalized linear interactive modeling (GLIM). The variability of the plotted points was not assumed to be constant. A Pearson goodness-of-fit test was performed for each individual data set. In the experiments shown below, significant lack of fit at the 5% level was found in 14 of 105 (13%) of data sets shown in Fig 4, and in 6% of those in later experiments. Those with significant lack of fit were not used for comparative purposes.

Direct comparison by limiting-dilution analysis of BM, umbilical cord blood, and leukapheresis product over 8 weeks in culture. The proportion of CAFC with time was compared directly in cultures of BM, umbilical cord blood, and leukapheresis product inoculated onto stromal layers derived from the same donor sample in six experiments from each group (n = 18, Fig 4). After an initial increase, the proportion of CAFC plateaued at 1:10^3 to 1:10^4 at weeks 3 to 6. The numbers of cobblestone areas then gradually decreased during weeks 6 to 8, especially in BM and umbilical cord blood. The proportion of CAFC per mononuclear cell at week 5 was 1:9,291 (1:3,296 to 17,602) in BM samples, 1:4,343 (1:3,857 to 15,209) in umbilical cord blood, and
tative experiments using for these data three groups. There was no statistically significant difference between the experiments products contained significantly more LTCIC at 8 weeks in 6 of culture only. For these experiments, five dilution steps = shown. The analytical method is described in complementary log-logs and the inoculum densities are expr-d as CAFC numbers plateau between weeks 3 and 6 of culture, in subsequent experiments CAFC were scored at week 5. GM-CFC numbers correlated with the presence of CAFC (Fig 5; r = .73, 95% confidence interval [CI] 0.32, 0.91). Trypsinization and manipulation of the cultures resulted in lower estimates of LTCIC obtained from GM-CFC than CAFC, with a mean difference of ~0.1, but this was not statistically significant (P = .11, two-tailed t-test). False negatives, ie, wells negative for cobblestone areas

1:7,590 (1:4,814 to 9,297) in peripheral blood stem cells. There was no statistically significant difference between the three groups (P = .7, Kruskal-Wallis test, n = 13). At week 8 of culture, the proportion of CAFC per mononuclear cell was 1:33,949 (1:13,692 to 127,582) in BM, 1:22,368 (1:17,904 to 75,466) in umbilical cord blood and 1:12,851 (1:8,127 to 14,537) in leukapheresis product. Leukapheresis products contained significantly more LTCIC at 8 weeks in these experiments (P = .01, Kruskal-Wallis test, n = 10).

Comparison of BM, umbilical cord blood, and leukapheresis product using a single estimate of LTCIC derived by limiting-dilution analysis. Because committed progenitors in the inoculum contribute to hematopoietic activity in the first 4 weeks of long-term cultures, and because estimated CAFC numbers plateau between weeks 3 and 6 of culture, in subsequent experiments CAFC were scored at week 5 or 6 of culture only. For these experiments, five dilution steps were used (each with 30 replicates) in the informative range. Using this extended data set (n = 41), the median calculated proportion of CAFC in unfractionated cells at week 5 of culture for 12 BMs was 1:13,314 mononuclear cells (range, 4,024 to 22,657), for 9 umbilical cord bloods 1:12,506 mononuclear cells (range, 3,857 to 21,244), and for 11 leukapheresis products 1:10,302 mononuclear cells (range, 2,252 to 52,060).

To determine whether similar estimates of LTCIC were obtained from total mononuclear cell and CD34+ cell frac-

tions, the population of CD34+ enriched cells obtained by selection on a Cephrate column were inoculated in a LTCIC assay at week 5 or 6, in nine patients in whom peripheral blood stem cells were mobilized with chemotherapy and administration of filgrastim. CD34+ cells constituted a median of 1.26% (range, 0.85 to 4.8) of mononuclear cells. The median calculated proportion of CAFC was 1:131 CD34+ cells (range, 76 to 443) and 1:8,175 mononuclear cells (range, 2,252 to 52,060).

Incidence of LTCIC estimated by clonogenic cells and CAFC. The production of GM-CFC in the supernatant and adherent layers of each individual well were measured in 5 BM, 4 umbilical cord bloods, and 5 leukapheresis product cultures at week 5. GM-CFC numbers correlated with the presence of CAFC (Fig 5; r = .73, 95% confidence interval [CI] 0.32, 0.91). Trypsinization and manipulation of the cultures resulted in lower estimates of LTCIC obtained from GM-CFC than CAFC, with a mean difference of ~0.1, but this was not statistically significant (P = .11, two-tailed t-test). False negatives, ie, wells negative for cobblestone areas

![Graph](https://via.placeholder.com/150)

Fig 2. Estimation of long-term culture-initiating cells by limiting-dilution analysis. Numbers of wells negative for cobblestone areas were scored. The proportions of negative wells are shown as complementary log-log lines and the inoculum densities are expressed as logarithms. The most informative range lies between the horizontal (---) lines. Dilution steps within this range have at least 90% of the information available from the optimal dilution step.11 Two representative experiments using 30 replicates at each of four dilutions are shown. The analytical method is described in the text. (A) PB, leukapheresis product; (B) NBM, BM. The Pearson goodness of fit value for these data sets is 0.73 and 0.77, respectively.

![Graph](https://via.placeholder.com/150)

Fig 4. Comparison of the proportions of cells generating hematopoietic foci from mononuclear cells in BM, umbilical cord blood, and leukapheresis harvest by limiting-dilution analysis. Preirradiated normal BM stroma from a single donor were used in each experiment. Six experiments from each group are shown. Estimated proportions of CAFC are shown at each week of culture. Each point represents the estimate made from 30 replicates at four dilutions. Only informative points are shown. (---) BM; (---) umbilical cord blood; (-----) leukapheresis product.
but positive for GM-CFC or vice versa, were rarely detected (total 6\%, Table 1).

In a single experiment of CD34\(^+\) selected cells (58 wells) assayed at 8 weeks of culture the proliferative potential of individual LTCIC was determined from the number of GM-CFC per well, as per Sutherland et al.\(^7\) The incidence of LTCIC estimated by CAFC was 1:157 and by GM-CFC 1:147. The number of colonies in positive wells ranged from 1 to 49 GM-CFC per well. The number of clonogenic progenitors per LTCIC was calculated to be 6:1.

**DISCUSSION**

This study describes the use of a simplified limiting-dilution assay in vitro to estimate the frequency of LTCIC as a measure of the putative stem cell in hematopoietic cells from different sources. We have confirmed in this system that the number of CAFC and GM-CFC are related to the number of cells inoculated so that both CAFC and GM-CFC can be used as endpoint measures in the three hematopoietic substrates (Fig 5). This does not necessarily imply that they are measuring the same stem cell population. Previous investigators have undertaken detailed studies using samples enriched by T-cell depletion and soya bean agglutinin\(^1\) or FACS subset analysis\(^2\)\(^,\)\(^3\) to characterize the stem cell subpopulations throughout the assay period. They have validated the assay of LTCIC as a measure of hematopoietic stem cells in mice, but definitive evidence is not available in humans. We have used CD34\(^+\) and unfractionated mononuclear cells to assess the proportion of LTCIC at weeks 5 through 6 of culture and have obtained similar estimates. These data also suggest that any accessory cells in unfractionated samples do not influence the progenitor cell population measured using this experimental design. Because these assays are very labor intensive, our observation that the assay can be simplified by inoculating unfractionated mononuclear cells and by scoring CAFC or GM-CFC at a single time point is of practical value.

Estimates of the proportion of LTCIC derived by limiting-dilution analysis are consistent with the description of hematopoietic activity obtained by measuring CFC production in standard long-term cultures. In the LTCIC assays, different progenitor cell populations contribute to hematopoiesis over the observation period.\(^8\)\(^,\)\(^11\) The dynamic nature of cobblestone areas in culture enables the contributions of the different progenitor populations responsible for short-term and longer-term engraftment to be assessed (Fig 4). This is consistent with previous studies of mice in which unfractonated and fractionated BM cells were simultaneously assayed in vivo and long-term culture initiating cell assays.\(^8\)\(^,\)\(^11\) The inoculated committed progenitors mature by week 4 and hematopoietic activity observed after this time is generated within the cultures by more primitive cells.

Our data confirm the observations of Sutherland et al.\(^7\) that at week 5 and week 8 different progenitor cell subpopulations are contributing to hematopoietic activity. Enrichment

**Table 1. Concordance Between Assessment of Well Positivity Obtained From CAFC and Week 5 GM-CFC**

<table>
<thead>
<tr>
<th>CAFC</th>
<th>GM-CFC</th>
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<tr>
<td>+</td>
<td>338 (34.8%)</td>
</tr>
<tr>
<td>-</td>
<td>20 (2%)</td>
</tr>
</tbody>
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N = 971 wells.
of early progenitors by selection of CD34+ cell subsets, CD331-15 HLA-DR\textsuperscript{high},\textsuperscript{14,16} rhodamine-123 dull,\textsuperscript{17} and 4-hydroperoxycyclophosphamide resistant\textsuperscript{14,16,18,19} are necessary to determine the contributions of the various cell populations to hematopoiesis at different time points and determine whether assessment at week 8 better represents the more primitive stem cell population than assessment at week 5. Even so, this methodology could still be usefully applied.

A variety of cytokines, alone and in combination, have been shown to mobilize hematopoietic progenitors into the circulation. The mix of different progenitors released may vary with the mobilizing regimen used. Numbers of GM-CFC and CD34+ cells are routinely used to assess the engraftment potential of BM and leukapheresis harvests.\textsuperscript{20} These parameters predominantly reflect clonogenic cell activity which might only be relevant to short-term hematopoietic reconstitution\textsuperscript{21} and not necessarily reflect the presence of a more primitive population capable of sustained engraftment.\textsuperscript{19}

The proportion of LTCIC in unstimulated peripheral blood and normal BM has been reported.\textsuperscript{7,14} Here we have shown that hematopoietic progenitors from BM, umbilical cord blood, and leukapheresis product behave similarly in culture. Increased hematopoiesis is caused by an increased proportion of primitive progenitors in the umbilical cord blood and leukapheresis products compared with BM. This is consistent with data showing that hematopoietic progenitors from mobilized peripheral blood and umbilical cord blood persist longer in long-term culture than BM cells.\textsuperscript{5,6} It is important to note that the leukapheresis products studied were obtained from patients undergoing induction chemotherapy, who had not been subjected to extensive pretreatment. Chemotherapy and G-CSF were used to mobilize progenitor cells into the peripheral blood. It would be unwise to extrapolate from these data to leukapheresis products obtained with different mobilizing regimens or from a heavily pretreated group of patients. At week 5 of culture, the proportion of LTCIC was about 1:10\textsuperscript{4} for all three hematopoietic sources. At week 8 this proportion was maintained in the leukapheresis product, however it fell in umbilical cord blood to 1.2 \times 10\textsuperscript{4} and in BM to 1.3 \times 10\textsuperscript{4}. Sutherland et al\textsuperscript{12} have reported the proportion of LTCIC in BM at week 5 to be approximately 1.2 \times 10\textsuperscript{5}. We have shown that plating efficiency is reduced by about 10\% between CAPC and colonies (Fig 5), which is the endpoint for the Canadian assay. Taking into account sample variability and differences in experimental design, these results are comparable.

At present, hematopoietic cells are mainly used for autologous and allogeneic transplantation. BM cells have long been used for both. In addition, hematopoietic stem cells are attracting interest as a source of cells for gene transfer and gene therapy. We have shown that umbilical cord blood and leukapheresis product are at least as good a source of LTCIC as BM for transplantation. The limiting-dilution analysis technique allows characterization of the diverse hematopoietic cell products and will assist in designing optimal mobilizing regimens.

In summary, the results of limiting-dilution analysis correlate well with hematopoietic activity in standard long-term culture and may be useful in characterizing the stem cell population. In both standard long-term culture and limiting-dilution analysis, the proportion of LTCIC in peripheral blood stem cells primed with chemotherapy and filgrastim is similar to or higher than that of BM and umbilical cord blood. The transplantation potential of primed peripheral blood stem cells and umbilical cord blood is at least as good as that of BM.

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**REFERENCES**

9. Ploemacher RE, van der Loo CM, van Beurden CAJ, Baert MRM: Wheat germ agglutinin affinity of murine hematopoietic stem cell subpopulations is an inverse function of their long-term repopulating ability in vitro and in vivo. Leukemia 7:120, 1993
14. Dooley DC, Law P: Detection and quantitation of long-term...


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