Use of Limiting-Dilution Type Long-Term Marrow Cultures in Frequency Analysis of Marrow-Repopulating and Spleen Colony-Forming Hematopoietic Stem Cells in the Mouse

By Rob E. Ploemacher, Johannes P. van der Sluijs, Carin A.J. van Beurden, Miranda R.M. Baert, and Pak Lam Chan

We have developed an in vitro clonal assay of murine hematopoietic precursor cells that form spleen colonies (CFU-S day 12) or produce in vitro clonable progenitors in the marrow (MRA cells) of lethally irradiated mice. The assay is essentially a long-term bone marrow culture in microtiter wells containing marrow-derived stromal "feeders" depleted for hematopoietic activity by irradiation. To test the validity of the assay as a quantitative in vitro stem cell assay, a series of unsorted and physically sorted bone marrow cells were simultaneously assayed in vivo and overlaid on the feeders in a range of concentrations, while frequencies of cells forming hematopoietic clones (cobblesstone area forming cells, CAFC) were calculated by means of Poisson statistics. Linear regression analysis of the data showed high correlations between the frequency of CFU-S day 12 and CAFC day 10, and between MRA cells and CAFC day 28. A majority of MRA activity and CAFC day 28 was separable from CFU-S day 12 and CAFC day 10. This correlation study validates the CAFC system as a clonal assay facilitating both the quantitative assessment of a series of subsets in the hematopoietic stem cell hierarchy and the study of single long-term repopulating cells in vitro.

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MATERIALS AND METHODS

Male CBA × C57BL/F1 mice, 12 to 30 weeks old, were either bred in the Central Animal Department of the Erasmus University (Rotterdam, The Netherlands) or purchased from the Medical Biological Laboratory ITRI (Rijswijk, The Netherlands) and maintained under clean conventional conditions. The drinking water was acidified to pH 2.8. In specific experiments mice were injected with 150 mg 5FU (Sigma, St Louis, MO) in phosphate-buffered saline (PBS) per kilogram of body weight in a lateral tail vein. Three or 6 days later they were killed and single cell suspensions prepared from their femurs and tibiae.

Sorting of bone marrow cells (BMC). BMC were prepared by cleaning femurs and tibiae from muscles and tendons and grinding them in a mortar using PBS. The cell suspensions were sieved over a nylon filter (mesh size 100 μm). To prepare cells that differed in their relative frequencies (either enrichment or depletion) of CFU-S day 7/8, CFU-S day 12 and MRA cells, we used fluorescence-activated cell sorting (FACS) after a pre-enrichment step that included either immunomagnetic bead-mediated negative selection of BMC lacking the expression of certain lineage-specific epitopes, or buoyant density centrifugation. For immunomagnetic...
bead-mediated selection, BMC were washed twice in Dulbecco's Modified Eagle's Medium (DMEM) and incubated for 1 hour on ice with the pooled supernatants of five hybridoma cell lines producing rat IgG antibodies to CD4 and CD8 determinants on T lymphocytes (antibodies H129.19 and 53.6.72, respectively), Mac-1 on monocytes, macrophages, granulocytic cells (antibody M1/70), B220 on B lymphocytes (antibody RA3.68B), and GR-1 on granulocytes (antibody RB6.8C5). After washing twice in DMEM containing 0.02% gelatin, the cells were incubated with goat-antirat IgG-coated paramagnetic beads (Advanced Magnetics, Cambridge, MA) in DMEM and 2% mouse serum and incubated for 30 minutes. The labeled cells were then withdrawn against an inner tube wall using a strong magnet, and the nonbound cells were collected. For buoyant density centrifugation a modification of a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient designed to isolate thymocytes and bone marrow subpopulations was used. The gradient was prepared at 4°C in 10 mL polycollomer centrifuge tubes (cat. no. 03124; Du Pont Cy, Wilmington, DE) with 3.6 mL of a bottom layer (1.078 g/mL), 2.4 mL of 1.069 g/mL and 1.2 mL of 1.055 g/mL, on top of which 1 mL of a BMC suspension was layered containing 2.5 to 3 × 10⁸ cells/mL. The gradients were spun at 23,500g for 30 minutes at 4°C using a Sorvall centrifuge (DuPont Instruments-Sorvall, DuPont Co, Newtown, CT) mounted with a HB-04 fixed-angle rotor. Cells from the interphase 1.069 to 1.078 g/mL were then collected, washed twice in PBS and 5% fetal calf serum (FCS), and properly diluted. Before sorting, the cells were either incubated with 0.1 μg/mL rhodamine-123 (Rh; Eastman Kodak, Rochester, NY) for 30 minutes at 37°C, or with 0.5 μg/mL fluorescinated wheat germ agglutinin (WGA-FITC; Polysciences, Warrington, PA) as previously described. Analysis and sorting of the cells was performed by a FACS II (B-D Systems, Becton Dickinson & Co, San Jose, CA) using a single argon laser at 488 nm. FITC and Rh fluorescence were measured using a 510-515 LP filter. After sorting, the WGA-labeled cell suspensions were added to equal volumes of 0.4 mol/L of the competitive sugar N-acetyl-D-glucosamine (Polysciences) in distilled water. The cells were not washed before intravenous injection to prevent loss of cells.

**Colony assays.** The day 7 and day 12 CFU-S content of cell suspensions was determined by injecting the appropriate dilutions into a lateral tail vein of lethally irradiated mice. Two opposing ⁶⁷⁷ Cs sources (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) were used to irradiate the recipient mice at a dose rate of 1.05 to 1.15 Gy/min with a total dose of 9.3 to 10.5 Gy. Control irradiated mice that did not receive cells were included for all observation days in each experiment. No endogeneous spleen colonies were found in the latter mice. Seven and 12 days later their spleen colonies were counted, fixed in Tellyesniczky's solution, and the macroscopic surface colonies counted.

**Quantification of colony-forming units in culture (CFU-C), including macrophage, granulocyte, and macrophage-granulocyte CFU.** was performed using a semisolid (0.8% methylcellulose; Methocel AP4 Premium, Dow Chemical, Rotterdam, The Netherlands) culture medium (α-modification of DMEM) in a fully humidified incubator at 37°C and 5% CO₂. The cultures contained 10% pokeweed mitogen-stimulated mouse spleen-conditioned medium (MSCM), 20% horse serum (HS), and 1% bovine serum albumin (BSA). Colonies were counted on day 7 of culture with an inverted microscope.

**Marrow repopulating ability (MRA).** The MRA describes the ability of a cell suspension to generate new BMC or CFU-S day 12 in the bone marrow of a lethally irradiated recipient mouse over a period of about 12 to 13 days. This period is determined by the survival of fatally irradiated mice that have not been grafted with BMC. Because we required a survival of at least four of five mice per group, the regeneration period was set to 12 days in this study. To measure MRA 12 days after injection of sorted or unsorted BMC into five lethally irradiated mice per group, different aliquots of their femoral marrow mixed mice were assayed for the presence of CFU-C. MRA was expressed as the number of CFU-C or nucleated cells per femur equivalent per 10⁷ cells injected. Control irradiated mice were included in each experiment and their endogeneous CFU-C (1 to 200 per femur) and cell number (2 to 4 × 10²⁷ per femur) on day 12 was used to correct experimental data.

**Long-term repopulating ability (LTRA).** LTRA of day 28 cobblestone areas was estimated using a sex-mismatched syngeneic chimeric model. The percentage of donor (male) type contribution to the peripheral blood leukocytes at 4 months posttransplantation into sublethally (8.25 Gy) irradiated syngeneic female hosts was measured with a fluorescent in situ hybridization technique using a Y-chromosome-specific probe.

**Micro-LTBM C technique.** LTBMC were performed as previously described. Briefly, flat-bottomed microrotifers (Costar, Badhoevedorp, The Netherlands) were inoculated with 5 to 10 × 10⁹ nonsieved BMC in 0.2 mL of LTBMC medium per well and an adherent stromal layer was grown in 9 to 11 days at 33°C, 10% CO₂, and 100% humidity. The layers were then irradiated (20 Gy) to eliminate hematopoietic activity without affecting the ability of the stroma to support hematopoiesis. One day later the medium was changed and various dilutions of fresh sorted or unsorted BMC were overlaid between 1 day and 6 weeks after irradiation to allow limiting dilution analysis (LDA) of the precursor cells forming hematopoietic clones under the stromal layers in these cultures. An LDA assay of a particular cell suspension included the use of six to eight dilution steps differing with a factor of 2 to 3 and 15 to 20 wells per concentration. Cultures were fed weekly by changing half of the medium. Between 3 and 28 days after overlay all wells were inspected with intervals of 1 or 2 days, and scored positive if at least one phase-dark hematopoietic clone (cobblestone area [CA], containing 5 to more than 10⁶ cells) was observed. The frequency of CA forming cells (CAPC) was then calculated using the maximum likelihood solution.

**CFU-C repopulating studies in micro-LTBM C.** Unfractionated BMC from normal mice, or from 5FU-treated mice at 3 days after injection, were overlaid in five parallel series on irradiated stromal layers in microrotifiers plates in six concentrations, starting with 81,000 cells/well down to 333 cells/well, the concentrations being threefold apart and using 20 wells per dilution. Cultures were fed weekly by completing a complete medium change and CFAC frequencies determined over a period of 4 weeks. On days 7, 14, 21, and 28 one of the parallel series was used for repopulating studies. The culture medium was removed from the wells and the adherent layers trypsinized by adding 0.05 mL of 0.5% trypsin for 1 to 3 minutes at room temperature. The digestion process was stopped by adding 0.1 mL of ice-cold FCS. The cells from 20 wells per concentration were pooled, and single-cell suspensions made by repeated passage through a 23-gauge needle. Cells were then centrifuged, taken up in 5 mL of medium and aliquots of the well contents were plated in the CFU-C assay. Recombinant human erythropoietin (Eprex; CILAG AG International, Switzerland; 2 U/mL) was added as additional growth factor, and colonies were counted on day 7. In some cases single colonies were picked on day 11, and cytopsins were stained with May Grünwald/Giemsa to allow the determination of differentiation lineages in each colony with light microscopy.

Statistical analysis was performed using the Statgraphics Vers. 3.0 program for MS-DOS computers (STSC, Inc and Statistical Graphics Corp, Rockville, MD).
CAFC ASSAY MEASURES HEMATOPOIETIC STEM CELLS

RESULTS

CA formation by various BMC populations. The kinetics of CA formation could be varied dramatically by sorting the cells on the basis of different criteria as previously shown. This is shown by Fig 1, which contains data from an experiment in which the light density fraction of BMC from 5FU-treated donor mice was subjected to FACS on the basis of Rh retention. The cells were sorted using forward and perpendicular light scatter thresholds that excluded most of the remaining granulocytes and part of the lymphocytes (blast window, ref 1). It appears that 5FU-day 6 bone marrow was far more enriched for CAFC day 28 (32×) than for CAFC day 6 (1.5×) as compared with their frequencies in normal BMC. The light-density fraction also showed an increasing enrichment for CA formation with increasing culture time. In these light-density fractions the frequency of CAFC day 6 was 10× as high as in unfractonated marrow, while CAFC day 28 were even 93× enriched over control BMC. Sorting out 10% of the most Rh-dull cells of this low-density preparation of 5FU BMC in the blast window (this window contained 30% of all nucleated cells sorted) gave a large depletion of CAFC day 5 (<1% of control BMC); however, CAFC day 28 were further enriched (154× over control BMC). Thus, this sorting protocol led to a CAFC day 28/CAFC day 6 enrichment ratio that exceeded 15,000 in a single cell suspension. In a less extreme way, 10% of the most Rh-bright cells in the lightscatter blast window were 113-fold enriched for CAFC day 17 while containing far lower numbers of precursors forming CAs on day 28 (threefold enrichment). These observations clearly indicate that early and late CAFC differ with respect to their sensitivity for the cytostatic agent 5FU, and their affinity for Rh. It is evident that the low Rh retention and a relative insensitivity for CAFC day 6 was 10× as high as in unfractonated marrow, while CAFC day 28 were even 93× enriched over control BMC. Sorting out 10% of the most Rh-dull cells of this low-density preparation of 5FU BMC in the blast window (this window contained 30% of all nucleated cells sorted) gave a large depletion of CAFC day 5 (<1% of control BMC); however, CAFC day 28 were further enriched (154× over control BMC). Thus, this sorting protocol led to a CAFC day 28/CAFC day 6 enrichment ratio that exceeded 15,000 in a single cell suspension. In a less extreme way, 10% of the most Rh-bright cells in the lightscatter blast window were 113-fold enriched for CAFC day 17 while containing far lower numbers of precursors forming CAs on day 28 (threefold enrichment). These observations clearly indicate that early and late CAFC differ with respect to their sensitivity for the cytostatic agent 5FU, and their affinity for Rh. It is evident that the low Rh retention and a relative insensitivity for CAFC day 6 was 10× as high as in unfractonated marrow, while CAFC day 28 were even 93× enriched over control BMC. Sorting out 10% of the most Rh-dull cells of this low-density preparation of 5FU BMC in the blast window (this window contained 30% of all nucleated cells sorted) gave a large depletion of CAFC day 5 (<1% of control BMC); however, CAFC day 28 were further enriched (154× over control BMC). Thus, this sorting protocol led to a CAFC day 28/CAFC day 6 enrichment ratio that exceeded 15,000 in a single cell suspension. In a less extreme way, 10% of the most Rh-bright cells in the lightscatter blast window were 113-fold enriched for CAFC day 17 while containing far lower numbers of precursors forming CAs on day 28 (threefold enrichment). These observations clearly indicate that early and late CAFC differ with respect to their sensitivity for the cytostatic agent 5FU, and their affinity for Rh. It is evident that the low Rh retention and a relative insensitivity for CAFC day 6 was 10× as high as in unfractonated marrow, while CAFC day 28 were even 93× enriched over control BMC. Sorting out 10% of the most Rh-dull cells of this low-density preparation of 5FU BMC in the blast window (this window contained 30% of all nucleated cells sorted) gave a large depletion of CAFC day 5 (<1% of control BMC); however, CAFC day 28 were further enriched (154× over control BMC). Thus, this sorting protocol led to a CAFC day 28/CAFC day 6 enrichment ratio that exceeded 15,000 in a single cell suspension. In a less extreme way, 10% of the most Rh-bright cells in the lightscatter blast window were 113-fold enriched for CAFC day 17 while containing far lower numbers of precursors forming CAs on day 28 (threefold enrichment). These observations clearly indicate that early and late CAFC differ with respect to their sensitivity for the cytostatic agent 5FU, and their affinity for Rh. It is evident that the low Rh retention and a relative insensitivity for 5FU of CAFC day 28 are features shared with MRA cells in vivo, while opposite properties characterize the CFU-S day 8 and day 12.

Correlation studies of in vivo- and in vitro-defined stem cell subsets. In a series of similar sorting experiments, data were collected that allowed the comparison of in vivo stem cell assays (CFU-S and MRA) with the in vitro LDA-type CAFC assay. The methods used for physical separation of the BMC have been described in Materials and Methods. Figure 2a confirms and extends earlier observations on a lack of correlation between the incidences of CFU-S day 12 and MRA[CFU-C] activity in a linear regression analysis (coefficient of determination $r^2$ = .236; n = 69), indicating that colony formation in the spleen and generation of new stem cells in the bone marrow are properties of physically largely separable stem cell subsets. It is evident that neither the frequencies of CAFC day 10 (Fig 2b; $r^2$ = .334; n = 53) nor those of CFU-S day 12 (Fig 2c; $r^2$ = .387; n = 51) show good correlation with the CAFC day 28 incidence, and that the average early and late CAFC significantly differ in a series of physical parameters. Comparison of the frequencies of all CAFC day types with those of CFU-S day 7 and 12 and the activity of MRA cells indicated three significant correlations. First, a high coefficient of determination ($r^2$ = .924; n = 55) in a linear regression analysis was observed between frequencies of CFU-S day 12 and CAFC day 10 (Fig 2d). When assaying low numbers of these stem cells (lower left side of Fig 2d), 6.7 CAFC day 10 were measured on a single CFU-S day 12, indicating that the in vivo seeding efficiency of CFU-S day 12 would be about 15% as approximated by this LDA assay in vitro. In the BMC fractions that were highly enriched for these stem cell types, 4.7 CAFC day 10 was scored on any CFU-S day 12, representing an in vivo seeding efficiency for CFU-S day 12 of about 21%.

A second remarkable high coefficient of determination ($r^2$ = .788; n = 53) was observed for the number of CFU-C generated in the marrow by stem cells with MRA (MRA[CFU-C]) and the frequency of CAFC day 28 in a variety of cell suspensions tested (Fig 2e). When the CAFC day 28 frequency was low, the data indicate that 0.18 CAFC day 28 equalled 10 CFU-C produced in one femur in a period of 12 days, ie, in this period the presence of 1 CAFC day 28 compared with a production of about 1,300 secondary CFU-C in the total marrow space of an irradiated mouse, accepting that one femur represents 16.6% of the whole marrow mass in these rodents. Differing with this was the calculated potency of highly enriched CAFC day 28 (right side of the fitted curve in Fig 2e) in which 1 CAFC day 28 equalled the generation of about 21,000 CFU-C in the total marrow in a 12-day period, whereas in unsorted marrow 1 CAFC day 28 was observed to correspond with 500 to 4,000 newly formed CFU-C. This lack of congruency in Fig 2e, in combination with good linear fit, may have been caused by the circumstance that a clonal assay (CAFC) is compared with the ability of a cell suspension to generate new CFU-C, irrespective of the number of precursors contributing to such a progenitor cell production. Apparently, the enrichment protocols used for MRA cells selected for a relative small population of potent CFU-C producers while excluding a majority of less capable MRA[CFU-C].

A third linear correlation ($r^2$ = .743; n = 36) was observed between the numbers of CAFC day 6 and those of...
CFU-S day 7 in the cell suspensions (Fig 2f). However, the limited number of data do not allow conclusive statistics on the validity of the CAFC assay as replacement for the in vivo CFU-S day 7 assay.

A full summary of the correlation coefficients calculated by linear regression analysis of the CAFC day 5 through 28 frequencies, and those of CFU-S day 7, CFU-S day 12 and MRA activity, is presented in Fig 3. The data indicate that the best approximation of actual CFU-S day 12 frequencies is done by counting CA between day 8 and 13 of culture. In addition, in the same cultures the frequency of CAFC day 28 showed a best linear fit with the in vivo activity of MRA cells. Furthermore, it appears that the CAFC assay is not fit to measure the CFU-S day 7 population as accurately as it does CFU-S day 12.

Presence of CFU-C in CAS. To investigate whether the potency of CAFC subtypes to generate secondary precursor cells differed with the length of the interval required for their clonal expression, micro-LTBMC cultures of different ages were investigated for their content of CFU-C. Because we have previously shown that the presence of replatable CFU-C in micro-LTBMC is strictly associated with that of CAS, the CFU-C content of a CAFC was estimated by dividing the number of replatable CFU-C per well by the number of CAFC per well as determined by limiting dilution analysis in the same cultures. From Fig 4 it appears that increasing numbers of normal BMC inoculated per well consistently decreased the number of secondary CFU-C detected per CA. Because large variability in replating data was observed when less than an average of 1 CA was detected per well, such data were excluded from this study.

Fig 3. Compilation of coefficients of determination ($r^2$) as approximated by linear regression analysis of the relation between the CAFC frequencies as determined in the day 5 through 28 period and the incidence of CFU-S day 7 (○), CFU-S day 12 (■), and the MRA(CFU-C) activity (□).

Fig 4. Effect of inoculum size on the CFU-C content of CAS as determined in the first 4 weeks of culture. BMC (1 to 81 x 10^3 per well) were overlaid on irradiated feeders and replated in a CFU-C assay on 4 consecutive weeks.
For these reasons, only data from series containing between 1 and 10 CAS per well were included in the comparative replating study presented in Fig 5. It is evident that normal bone marrow-derived CAS present between week 1 and 3 only contained 10 to 20 CFU-C, while 50 CFU-C were detected in the few CAS present on week 4. These data suggest that the average CAFC day 28 has a higher ability than earlier detectable CAFC to produce in vitro clonable progenitors. Except for week 4, CAFC in bone marrow from 5FU-treated mice contained consistently more CFU-C than CAFC in control marrow, suggesting that 5FU is selectively toxic for less potent CAFC.

In normal bone marrow, CAFC replated from day 28 CAS formed a higher percentage of mixed colonies (GEM, GEMMeg) than CFU-C from day-7 CAS (Table 1). In bone marrow from 5FU-treated mice both CAFC day 7 and CAFC day 28 generated a relatively high percentage of CFU-Mix. These data again support the assumption that CAFC day 28 in normal marrow, and most CAFC in 5FU marrow, are more primitive than CAFC giving rise to early CA formation in normal marrow.

Presence of stem cells in day 28 CAS. From preliminary experiments it was clear that the content of in vivo long-term repopulating stem cells (LTRA), CFU-S day 12, and CAFC day 10/28 in single day-28 CAS varies widely. To obtain a representative estimate of the content of the more primitive stem cells in day-28 CAS, each well in 40 96-well plates, containing irradiated marrow-derived stroma, was seeded with 3,000 normal BMC and the medium was changed weekly. Nine percent of the wells contained CAS at day 28, indicating that the probability of finding a single clone per CA-containing well was higher than 95% according to Poisson statistics. One hundred CA-containing wells were then pooled and aliquots equalling either one, two, or four CAS were injected into lethally irradiated recipient mice, and macroscopic spleen nodules were counted on day 12. Part of the pooled CA-containing wells was overlaid in the micro-LTBM assay (eight dilutions of 15 wells each, twofold apart, starting with an aliquot of four CA-containing wells per well). These experiments indicated that an average day-28 CA contained 2.2 CFU-S day 12, 15.6 CAFC day 10, and 0.29 CAFC day 28. In similar tests using identical numbers of empty wells we did not detect replatable CFU-S day 12 or CAFC.

In vivo LTRA was estimated by pooling 200 CA-containing wells on day 28 and injecting one fifth of this suspension IV into each of five syngeneic female mice per group that had been conditioned with a sublethal irradiation dose of 8.25 Gy. Four months later the male donor cells constituted 0%, 2%, 9%, 15%, and 16%, respectively, of all nucleated cells in the peripheral blood, i.e., three of five mice showed a significant donor-type signal. We did not find donor-type repopulation in female mice that received an equivalent of 40 empty wells on day 28.

**DISCUSSION**

To obtain statistical support for our earlier suggestion, that the time-dependent CA formation in LTBMC reflects the turnover and primitiveness of CAFC, we have performed a comprehensive study on the qualitative and quantitative relation between in vivo and in vitro stem cell assays. This study was facilitated by the application of purification protocols that give extreme variations in the relative frequencies of the various HSC subsets. The combined use of 5FU and FACS on the basis of light scatter, affinity for wheat germ agglutinin, and Rh retention indeed allowed the acquisition of subset ratios exceeding several hundred-fold.\(^{14,22}\)

The data present evidence that the CAFC day-10 assay accurately measures CFU-S day 12, irrespective of the presence or absence of cells with MRA activity. The regression analysis also showed that 4.7 to 6.7 CAFC day 10 are detected on every CFU-S day 12, suggesting an in vivo spleen seeding efficiency for CFU-S day 12 of 15% to 20%. This high figure can be explained by assuming that CFU-S day 12 have either a higher seeding in the irradiated spleen than the 5% to 12% as previously established for CFU-S day 7\(^{15,16}\) and CFU-S day 12\(^{17}\) using in vivo retransplantation studies, or the cloning efficiency of the CAFC assay was lower than 100%.

A fairly good linear fit was observed for the CAFC day 28 and the narrow repopulating activity of the cell suspensions. The suggestion of Reincke et al\(^{18}\) that murine stem cells giving rise to day-28 clones in LTBMC are indicative of CFU-S activity, is thus not supported by our data. It should be noted that the present data have been plotted in an absolute way, which leads to significantly lower correlation coefficients than observed when comparing enrichments for these HSC subsets in single experiments. Although the

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**Table 1. Differential Capacity of CFU-C Replated From CAS on Day 7 or 28 of Culture**

<table>
<thead>
<tr>
<th>Source of Bone Marrow Cells</th>
<th>Day of Replating</th>
<th>% M/GM Colonies</th>
<th>% GEM/GEMMeg Colonies</th>
<th>No. Colonies Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>96</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>5FU</td>
<td>7</td>
<td>78</td>
<td>22</td>
<td>156</td>
</tr>
<tr>
<td>Control</td>
<td>28</td>
<td>83</td>
<td>17</td>
<td>276</td>
</tr>
<tr>
<td>5FU</td>
<td>28</td>
<td>74</td>
<td>26</td>
<td>188</td>
</tr>
</tbody>
</table>

Data represent percentages of CFU-C-derived colonies containing combinations of monocyte/macrophage (M), granulocyte (G), erythroid (E), or megakaryocytic (Meg) differentiation lineages.
linear regression analysis showed excellent fit, the ratio of CFU-C generated per femur and the frequency of CAFC day 28 as assayed by LDA increased with the increasing presence of these cells in the cell suspensions tested, leading to a deviation of the congruency line. To comprehend this it is recalled that the MRA assay is a nonclonal in vivo regeneration assay, and that a selection for more potent MRA cells using the protocols applied may thus give higher enrichments for in vivo activity than for clonogenic capacity in the CAFC test. This notion is supported by our observation that in vitro the number of CFU-C produced per CAFC may vary considerably with the protocol used to obtain the cells (Fig 5).

In conjunction with the regression data, the primitive characteristics of CAFC day 28 is also apparent from the observation (Fig 5, Table 1) that these stem cell subsets produce more CFU-C than cells forming early detectable CAFs, and that relatively more CFU-C-derived colonies contain three or more differentiation lineages. The absolute frequency of CAFC day 28, ie, 1 to 4 per 10⁶ cells in normal marrow, is in line with earlier reports on the incidence of extensively proliferating, or long-term repopulating, HSC in mice and humans, and shows that the CFU-S and CFU-C assays significantly overestimate the incidence of stem cells with MRA and LTRA.

Although good linear fit is shown between frequencies of hematopoietic clones in vivo and in vitro on particular days, and the clone-initiating cell may have quite primitive characteristics, our correlation data do not imply that a single colony (either in the spleen or in a culture well) consequently still contains primitive HSC after weeks of culture. Conditions for obtaining single clones in liquid LTBM C can only be created in a statistical manner, ie, the percentage of positive wells should ideally be kept lower than 10% to give a higher than 95% probability that a positive well contains not more than one clone. Although preliminary experiments have shown that not every single clone includes secondary CFU-S and CAFC, pooled single clones at day 28 of culture appear to contain in vivo LTRA cells, CFU-S day 12, and CAFC day 10 and day 28, suggesting that at least part of CAFC day 28 should be classified to the most primitive HSC subset identified today. Since CAFC day 28 have gone through 3 to over 20 divisions in order to generate a day 28 cobblestone area, such individual colonies are unlikely to all contain detectable primitive stem cells.

The murine CAFC assay offers an attractive alternative for the costly and animal-requiring in vivo HSC assays. Secondly, when the assay is read during 4 weeks, it supplies the observer with a full cross-section of HSC subset frequencies in a particular cell suspension, ranging from transient spleen colony-forming cells to the primitive MRA cells. This is of particular importance when quantitative information is required on the life-sparing capacity of a graft, which should contain both a sufficiently large number of rapidly proliferating CFU-S responsible for short-term survival and stem cell-generating MRA cells that may have a delayed onset in proliferative activity and therefore have low radioprotective ability. Such a delayed initiation of clonal expansion in vitro is characteristic of cell suspensions enriched in MRA, or depleted for CFU-S, as is the case in BMC from 5FU-treated mice and in Rh-dull BMC. Finally, the availability of the CAFC system should facilitate studies of molecular events associated with the regulation of proliferation and differentiation of single LTRA cells.

The present data encourage further study on the validity of an LDA-type LTBM C, both in humans and in mice, as a clonal assay for enumeration of cells that determines the success of bone marrow transplantation. In mice, such long-term engraftment studies using sex-mismatched donor-host combinations or polymorphism for T-200/Thy-1 are currently performed in our laboratory.

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