An In Vitro Limiting-Dilution Assay of Long-Term Repopulating Hematopoietic Stem Cells in the Mouse

Rob E. Ploemacher, Johannes P. van der Sluijs, Jane S.A. Voerman, and Nicolaas H.C. Brons

We have developed a limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse using a miniturized stroma-dependent bone marrow culture assay in vitro. The cells were overlaid on irradiated stromal layers in microtiters wells in a range of concentrations, and frequencies of cobblestone area-forming cells (CAFC) were calculated by employing Poisson statistics. The production of secondary granulocyte/macrophage colony-forming units (CFU-G/M) in the adherent layer of individual wells was correlated with the presence of such cobblestone areas. CAFC frequencies were determined in bone marrow cell suspensions that were either enriched for marrow repopulating ability (MRA) in vivo, while depleted for spleen colony-forming units (CFU-S), or vice versa. The separation of bone marrow cells (BMC) was either based on centrifugal elutriation, or monoclonal antibody-mediated magnetic depletion of cells carrying cell surface differentiation antigens, and subsequent sorting on the basis of light scatter and rhodamine-123 retention as a measure of mitochondrial activity. In addition, 5-fluorouracil-resistant BMC were studied. Our investigations show that a time-dependent cobblestone area formation exists that reflects the turnover time and primitiveness of CAFC. The frequency of precursors forming cobblestone areas on day 28 after overlay is proposed to be a measure for MRA, whereas the day-7 CAFC frequency closely corresponds with day-12 CFU-S numbers in the suspensions tested.

EVIDENCE IS INCREASING that the hematopoietic stem cell compartment is extremely heterogeneous, and represents a hierarchy of primitive cells on the basis of decreasing ability to generate new stem cells, decreasing proliferative potential and pluripotentiality, and increasing turnover rate. Thus, the most primitive stem cells generate many spleen colony-forming units (CFU-S), and cells that ensure survival of fatally irradiated recipients, in the irradiated bone marrow in vivo.1 This marrow repopulating ability (MRA) is largely associated with resting cells as evidenced by the increased MRA of cells surviving the cytotoxic agents 5-fluorouracil (5-FU),2 bromodeoxyuridine,3 or 5-hydroxyuracil.4,5 Furthermore, in contrast to CFU-S and in vitro clonal progenitor cells, these MRA cells, or pre-CFU-S, have a low mitochondrial mass per cell as suggested by the minimal retention of the supravital fluorochrome rhodamine-123 (Rh).6,7

Until now it has not been possible to determine the frequency of MRA cells in cell suspensions because the in vivo assay for MRA measures the total number of CFU-S generated on a basis of the number of cells injected without yielding information about the number of precursors contributing to the total number of CFU-S formed. Unfortunately, any attempt to perform limiting-dilution assays in vivo on a regular basis is prohibited by the large numbers of animals required. In addition, colony formation of 5-FU-resistant or Rh-dull cells in semisolid media is poor.

Recently, the notion emerged that CFU-S give only short-lived, stroma-associated hematopoiesis in vitro.4,10 We have demonstrated that the ability of a stem cell for long-term engraftment of an irradiated stromal layer in flask cultures is inversely related to its mitochondrial activity, and corresponds well with its MRA in vivo.11 In addition, we have proposed that granulocyte/macrophage colony-forming unit (CFU-G/M) production in long-term bone marrow culture (LTBMC) at 1 or 4 weeks after overlay of test cells can be taken as a semi-quantitative measure of the number of inoculated CFU-S or MRA, respectively.11

In view of these observations, we have set out to develop a quantitative in vitro assay that permits direct measurement of the frequencies of hemopoietic stem cells responsible for short- or long-term hemopoietic engraftment both in vivo and in vitro. It has been demonstrated earlier that clonal analysis using a limiting dilution assay in Dexter-type cultures is feasible, however limited by the large numbers of flasks that have to be analyzed.12 The present assay has been designed to meet requirements for a simple and unequivocal endpoint, routine use, and a sufficient number of cultures to permit reliable Poisson statistics.

MATERIALS AND METHODS

Male CBA X C57BL/F1 mice, 12 to 30 weeks old, were purchased from the Medical Biological Laboratory TNO (Rijswijk, The Netherlands) and maintained at the Laboratory Animals Center under clean conventional conditions. The drinking water was acidified to pH 2.8. In specific experiments, mice were injected with either 150 or 75 mg 5-FU (Sigma, St Louis, MO) in phosphate-buffered saline (PBS)/kg of body weight in the lateral tail vein. Three days later they were killed and single-cell suspensions prepared.

Sorting of bone marrow cells. Bone marrow cells (BMC) were prepared by cleaning femurs 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). In order to prepare cells that were either enriched for MRA cells and depleted for CFU-S, or vice versa,3 we used fluorescence-activated cell sorting of BMC following a pre-enrichment step that included either centrifugal elutriation13 or paramagnetic bead-mediated negative selection of cells lacking the expression of lineage-specific markers.13 RPMI medium with 0.4% bovine serum albumin (BSA, Sigma, St Louis, MO), penicillin (100 IU/mL), and streptomycin (100 mg/mL) was used as standard counter current centrifugal elutriation (CCE) medium. For paramagnetic bead sorting cells were washed twice in Dulbecco's Modi-

From the Department of Cell Biology II, Erasmus University, Rotterdam, The Netherlands.

Submitted March 14, 1989; accepted July 24, 1989.

Address reprint requests to Rob E. Ploemacher, PhD, Department of Cell Biology II, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.
0006-4971/89/7408-0127$3.00/0
fied Eagle's Medium (DMEM), and incubated for 1 hour on ice with the pooled supernatants of five hybridoma cell lines producing rat immunoglobulin G (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 RA3.6B2), and GR-1 (on granulocytes; antibody RB6.8C5). After washing twice in DMEM containing 0.02% gelatin, the cells were incubated with goat-anti-rat IgG-coated paramagnetic beads (Advanced Magnetics, Cambridge, MA) in DMEM and 2% mouse serum and incubated at 0°C for 30 minutes. The labeled cells were then withdrawn against an inner tube wall using a strong magnet, and the nonbound cells were collected. These cells will hereafter be called Lin<sup>-</sup> cells to designate the absence of expression of a series of lineage markers. Following CCE, or paramagnetic bead sorting, cells were incubated for 45 minutes at 37°C in DMEM containing 2 μg/mL Rh (Eastman Kodak, Rochester, NY). In order to remove the excess of Rh, the cells were incubated for another 30 minutes in DMEM at 37°C. Finally, the cells were washed twice.

Analysis and sorting of the Rh-labeled cells was performed by a FACS 11 (B-D Facs Systems, Becton Dickinson & Co, Sunnyvale, CA) with an argon laser set at 488 nm. The cells from the E (11 to 12 mL/min) irradiation preparations, or the Lin<sup>-</sup> cells, were sorted into two fractions differing in Rh-retention (Rh-dull containing 10% of cells with little Rh uptake; and Rh-bright cells, which included 10% of the most brightly fluorescent cells) within a light scatter window. The lower bound of the forward scatter was set to include approximately half of the lymphocytic cells, while most of the granulocytes were excluded on the basis of perpendicular light scatter ("blast cell window"). This light scatter window was set using Rh-labeled unfractionated BMC as a reference.

**Colony assays.** The day-8 and day-12 CFU-S content of cell suspensions was determined by injecting the appropriate cell dilutions into a lateral tail vein of lethally irradiated mice. The dilutions used were estimated from previous experiments to give between one to five surface nodules on day 8 and between zero to six on day 12 to avoid confluency. Two opposing cesium-137 sources (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) were used to irradiate the recipient mice at a dose rate of 1.15 Gy/min with a total dose of 9.9 to 10.5 Gy. Control irradiated mice that did not receive cells were included for all observation days in each experiment. Eight or 12 days later their spleens were excised, fixed in Telleyeniczky's solution, and the macroscopic surface colonies counted.

Quantification of CFU-G/M (CFU-M, CFU-G, and CFU-GM) was performed using a semisolid (0.8% methylcellulose; Methocel AP4 Premium, Dow Chemical, Rotterdam, The Netherlands) culture medium (α-modification of DMEM) at 37°C and 5% CO.<sub>2</sub>. The cultures contained 20% con-A-stimulated mouse spleen conditioned medium (MSCM), 10% fetal calf serum (FCS), and 1% BSA. Granulocyte-macrophage colonies were counted on day 7 of culture with an inverted microscope. In the study of the in vitro clonogenic properties of Rh-dull and Rh-bright cells the MSCM was replaced by 10% pokeweed-mitogen–MSCM, and 1 IU sheep erythropoietin/mL (Connaught Laboratories, Ontario, Canada) and 2 × 10<sup>4</sup> U of human recombinant interleukin-6 (rIL-6) (purified from *Escherichia coli*)/mL (Dr L. Aarden, CLB, Amsterdam, The Netherlands) were included in the medium. These growth factor concentrations are optimal for colony formation in the system used.

**Marrow repopulating ability.** After injection (13 days) of unfractionated or sorted cells into five lethally irradiated mice, aliquots of their marrow cells were assayed for the presence of CFU-S-12 and CFU-G/M. Marrow repopulating ability was expressed as the number of CFU-S-12 or CFU-G/M per organ equivalent per 10<sup>5</sup> cells injected. Control irradiated mice were included in each experiment and were never found to have endoge-
Table 1. Colony-Forming Ability in Methylcellulose of Cells Separated on the Basis of Differences in Rh Retention

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Colonies Counted on Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elutriated BMC</td>
<td>75-210 57-230 150-285</td>
</tr>
<tr>
<td>CCE/Rh-dull</td>
<td>0                  0-13  26-59</td>
</tr>
<tr>
<td>CCE/Rh-bright</td>
<td>850-2,000 672-19,000 843-19,000</td>
</tr>
</tbody>
</table>

Data represent the range of the colony number/10⁶ cells plated in three separate experiments. Cultures were stimulated with PWM-MSCM, erythropoietin, and rIL-6.

*Sorted in the light scatter blast cell window from elutriated BMC.

Small colonies on top of the stroma within the first 3 days. As of day 2 single cells were observed in between or fully beneath the stromal cells as judged from their lack of refractiveness in phase-contrast and their large diameter, which was indicative of flattening. As of day 3 such stromal-covered cells were observed to have proliferated and formed clusters of often tightly packed cells that are referred to as cobblestone areas (CA). Some CA kept on growing for a few weeks or months and then contained many thousands of cells, and no cells in that area were observed to have migrated up to the surface to become differentiated granulocytes and monocytes/macrophages. Light microscopy of the cells collected from such wells by trypsinization indicated the presence of many blast cells. Other CA disappeared either after a few days or weeks by migrating to the upper surface of the stromal layer while losing their phase density. In a few days these cells differentiated, acquired the light halo-effect of spherical cells and then migrated away over the stromal surface (Fig 1). Remarkably, no cells were found in the culture medium throughout the culture period.

Choice of endpoint in micro-LTBMC using limiting-dilution strategy. From Table 2 it appears that no nonadherent CFU-G/M were found in single wells with extensive hemopoiesis including the presence of CA. The demonstration of replatable CFU-G/M was strictly associated with the presence of CA in any well tested, but not with stroma-adherent hematopoietic cells on the interface with the medium. On the basis of these data, and the consistent observation that cells contained within the stromal layer are associated with actively proliferating and more primitive hematopoietic cells, we decided to use the presence of CA in a well as an endpoint for limiting-dilution-type assays. Subsequently, we tested whether the number of BMC cells overlaid on preirradiated layers in micro-LTBMC correlated with the occurrence of CA as an endpoint. To this purpose we overlaid the wells with a series of dilutions ranging from one cell to 80,000 cells per well, and using 20 to 30 wells per dilution. Microtiter plates were repeatedly screened over a period of 32 days under phase contrast, and wells were scored positive when at least one CA containing five cells or more was encountered. A typical experiment is presented in Fig 2. It is clear that the percentage of negative wells in any dilution series is strictly determined by the number of cells inoculated per well. In conjunction with the association of replatable CFU-G/M this observation provides the validity of the CA as endpoint for limiting-dilution assays of primitive hematopoietic stem cells.

Table 2. Localization of Replatable CFU-G/M in Micro-LTBMC

<table>
<thead>
<tr>
<th>Component Tested</th>
<th>No. of Wells Tested</th>
<th>No. of Wells Containing CFU-G/M</th>
<th>CA</th>
<th>Hematopoietic Cells on Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>80</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adherent layer</td>
<td>35</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adherent layer</td>
<td>46</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Adherent layer</td>
<td>30</td>
<td>18</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Adherent layer</td>
<td>81</td>
<td>69</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Replated between seven and 28 days after overlay.

Abbreviations: +, present; -, absent.

Fig 1. Phase contrast micrograph of differentiating clones on day 10 in a micro-LTBMC of unseparated bone marrow. Maturing hematopoietic cells appear as small refractive (light) cells on the interface of stromal cells and the supernatant. The dark cells are cobblestone cells, which are covered by the adherent stromal layer (original magnification ×250).
such that a fraction of cultures at a certain observation time would not contain any cobblestone area-forming cells (CAFC). From the fraction of nonresponding cultures, using the Poisson equation, it was then possible to calculate the CAFC frequency. From experiments presented in Fig 2 we have calculated average CAFC frequencies in normal murine bone marrow. As is clear from Table 3 and Fig 3, precursors forming CA between days 5 and 7 after overlay (CAFC-5d and CAFC-7d) occur with highest frequencies in the marrow, while precursors for later CA formation occurred with decreasing frequencies.

Distribution of CAFC frequencies in cell suspensions differing in Rh retention. In order to investigate any heterogeneity in CAFC scored after different periods of culture, we overlaid stromal layers in microcultures with cells sorted on the basis of differences in mitochondrial mass/cell as indicated by their Rh retention. As reported earlier, Rh-bright cells contain many CFU-S on days 7 and 12, but no cells that generate new day-12 CFU-S and CFU-G/M in vivo, ie, cells with MRA. Conversely, Rh-dull cells are depleted for CFU-S, but contain most of the MRA.

Table 3. Comparative Precursor Cell Frequencies in BM

<table>
<thead>
<tr>
<th>Precursor Type</th>
<th>Frequency/Range</th>
<th>No. of Precursors/10⁶ Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-G/M</td>
<td>1/385-710</td>
<td>205 (20) 140-260</td>
</tr>
<tr>
<td>CFU-S-7d</td>
<td>1/100-265</td>
<td>675 (73) 375-1,000</td>
</tr>
<tr>
<td>CFU-S-12d</td>
<td>1/100-265</td>
<td>725 (110) 375-1,000</td>
</tr>
<tr>
<td>CAFC-3d</td>
<td>1/990-3,300</td>
<td>59 (17) 30-101</td>
</tr>
<tr>
<td>CAFC-5d</td>
<td>1/170-1,150</td>
<td>259 (67) 87-588</td>
</tr>
<tr>
<td>CAFC-7d</td>
<td>1/205-500</td>
<td>339 (30) 200-488</td>
</tr>
<tr>
<td>CAFC-14d</td>
<td>1/1,110-2,500</td>
<td>68 (8) 40-90</td>
</tr>
<tr>
<td>CAFC-21d</td>
<td>1/3,200-15,000</td>
<td>17 (3) 7-31</td>
</tr>
<tr>
<td>CAFC-28d</td>
<td>1/9,000-50,000</td>
<td>6 (1) 2-11</td>
</tr>
</tbody>
</table>

*Stimulated by 10% PWM-MSCM.
†Corrected for seeding efficiency (tₚ₉ hours = 4%).
‡CAFC frequency data from 12 separate experiments. CFU-S and CFU-G/M data have been collected over the last 3 years in our laboratory.

By limiting dilution (Figs 4A and B), Rh-bright cells appeared to have high frequencies of CAFC-7d, but low frequencies of cells forming late CA (eg, CAFC-28d). In contrast, Rh-dull cells formed far more late CA and fewer early CA than unfractionated and Rh-bright BMC. These data clearly indicate that early and late CAFC differ with respect to their mitochondrial activity. Table 4 shows the agreement in frequency ratios between the various cell suspensions as measured by the in vitro CAFC assay and the in vivo acquired data for CFU-S and MRA cells. Apparently, the differences between frequencies for CAFC-5d and CAFC-7d in the various cell suspensions are comparable with the CFU-S numbers, whereas CAFC-28d frequencies correlate with data for MRA.

Distribution of CAFC frequencies in bone marrow from 5-FU-treated mice. The hematopoietic stem cell compartment in the mouse is structured as a concatenated series of stem cells with progressively limited proliferative potential, and increasing turnover rate and maturity. Thus, treatment of mice with 5-FU reduces CFU-GM, and cells forming spleen colonies in irradiated mice on days 8 and 10 (CFU-S-8 and CFU-S-10) considerably more than CFU-S-13, which in turn are affected far more than MRA cells.

We have measured the CAFC frequency in the bone marrow of mice injected 3 days previously with either 150 or 75 mg 5-FU/kg BW. Figure 5 shows that treatment with 150 mg 5-FU depleted the femoral content of early CAFC (CAFC-5d to CAFC-9d), but increasingly spared the precursors of later appearing CA. Precursor cells responsible for CA formation after day 21 were not reduced by the high-dose 5-FU, indicating their essentially noncycling status. As compared with control mice, femurs of mice injected with 75 mg/kg BW three days before contained even more precursors that formed CA after 3 weeks of culture. It appears, therefore, that the cohort of CAFC differing in time-dependent CA formation reflects the above mentioned hierarchy of hematopoietic precursors on the basis of turnover rate and ability to generate new stem cells. Figure 6 comprises the effect of 150 mg 5-FU/kg BW on the femoral content of
Fig 3. Distribution of day 3 to day 28 CAFC frequencies in normal bone marrow. Data are the arithmetic mean of 12 individual experiments (bars, 1 SEM).

Fig 4. Distribution of CAFC frequencies as scored on various days after overlay of BMCs, pre-enriched by means of centrifugal elutriation (A) or paramagnetic beads (B), and sorted on the basis of differences in Rh retention. (△), Pre-enriched BMC; (□), Rh-bright cells; (○), Rh-dull cells.
can be seen that both CFU-G/M and CFU-S-8 are depleted approximately 4% to 7% of early CAFC reflect those of CFU-G/M and CFU-S.

Table 4. Frequencies of Short- and Long-Term Repopulating Stem Cell Subsets In Vivo and In Vitro

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>CAFC/10^4 Nucleated Cells</th>
<th>CFU-S/10^5 Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 7</td>
</tr>
<tr>
<td>Unfractionated BMC</td>
<td>248</td>
<td>245</td>
</tr>
<tr>
<td></td>
<td>(62)</td>
<td>(53)</td>
</tr>
<tr>
<td>CCE/Rh-dull</td>
<td>47</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(31)</td>
</tr>
<tr>
<td>CCE/Rh-bright</td>
<td>4,044</td>
<td>7,800</td>
</tr>
<tr>
<td></td>
<td>(1,469)</td>
<td>(1,848)</td>
</tr>
</tbody>
</table>

Numbers are arithmetic mean of four experiments (1 SE).
*tNot corrected for seeding efficiency.
†Number of CFU-S day 12 contained in a femur of an irradiation recipient on a basis of 10^4 cells injected, determined at day 13.
‡Sorted from elutriated BMC within the light scatter blast cell window.

CAFC, CFU-GM, CFU-S days 8 and 12, and MRA cells. It can be seen that both CFU-G/M and CFU-S-8 are depleted to less than 1% following this 5-FU protocol, whereas only approximately 4% to 7% of CFU-S-12 and 30% to 60% of MRA cells survive. These data confirm that frequencies of early CAFC reflect those of CFU-G/M and CFU-S, whereas late CAFC frequencies correlate with those of MRA cells. The observation that the CAFC-28d represents a hematopoietic precursor cell type which is not affected by 5-FU treatment suggests that this precursor is the most primitive subset of cells able to repopulate the irradiated bone marrow.

**Half-life of CA.** During the first days after overlay of cells on the stroma, developing CA outnumber disappearing hematopoietic clones, leading to a maximum CA frequency. Hereafter, a process of clonal extinction can be observed in time that is characteristic for the cell type studied. Table 5 shows the half-life for CA observed in cultures of Rh-bright and Rh-dull BMC, 5-FU-resistant BMC, and normal BMC during this extinction process. Short half-lives for CA evoked by Rh-bright cells, and long values for Rh-dull BMC, and BMC from mice 3 days after 5-FU injection, are evident.

**DISCUSSION**

This study describes an in vitro limiting-dilution assay for estimating the frequency of a series of hematopoietic stem cells that give rise to time-dependent clonal amplification under the stromal layer in LTBM. We propose the term cobblestone area-forming cells, or CAFC, for these primitive cells. An important implication of the presented data is, that the frequency required for these primitive cells to repopulate stromal layers directly relates to (1) their turnover time as indicated by their resistance to 5-FU, (2) to their ability to generate secondary CFU-S and CFU-G/M both in vitro and in vivo, and (3) inversely relates to their mitochondrial activity. This concept is compatible with a previously proposed model considering bone marrow functionality organized as a concatenated series of stem-cell compartments in which turnover time decreases as maturity increases. Time course studies of colony formation by stem cells proliferating either in vivo or in vitro have revealed a positive correlation between the onset of growth, and the extent of self-renewal subsequently observed. We propose therefore on the basis of the available information that the frequency of the various subsets belonging to this stem cell hierarchy can be quantified by scoring CAFC frequencies as a function of time after overlay on stroma layers in vitro.

The present report introduces a frequency analysis assay in vitro of stem cells associated with marrow repopulating ability. MRA cells have been shown to have a high capacity for the generation of secondary CFU-S day 12 and cells with radioprotective ability and CFU-G/M both in vitro and in vivo. As demonstrated in Table 1, colony formation of Rh-dull cells in semisolid medium was poor, and contrasted with their ability for long-term repopulation of irradiated mice and stromal layers in vitro. It should be noted that Rh-dull cells did only give rise to small colonies, even in the presence of rIL-6 and interleukin-3 (IL-3) (contained in PWM-MSCM). IL-6 and IL-3 have been described to act
synergistically to hasten the appearance of multilineage blast cell colonies. Up to the present the MRA of a cell suspension could only be determined in vivo, but frequency analyses were impracticable due to the large number of animals required for the limiting-dilution technique. In addition to the use of this micro-LTBMC for MRA cell frequency analysis, Table 4 and Fig 4 clearly show that a good indication of the CFU-S--d-12 frequency can be obtained from limiting-dilution analysis of CAFC d-7. The extinction of the majority of normal BMC-derived clones (see Table 5) in our miniaturized LTBMC assay is fully compatible with the observation that the total life of individual clones in flask cultures is between 3 and 15 days. The rapid decay of hematopoietic clones generated by Rh-bright cells also firmly supports our earlier observation21 that such cells form a majority of transient erythrocytic spleen nodules in an irradiated recipient.

Although scoring of CA as an endpoint in the presently described limiting-dilution analysis is very time-consuming, it presents as an unambiguous measure of proliferative activity. It is superior to the scoring of hematopoietic cell clusters on top of the layer, because such cells are more mature and may have lost their proliferative activity days before the time of observation. It has been consistently observed that the actively proliferating and more primitive hematopoietic cells are preferentially located within the adherent layer, whereas with increasing maturity the cells migrate to the surface of the layer and into the growth medium. Moreover, CFU-S in the adherent layer have been reported to have a significantly higher self renewal than have the nonadherent CFU-S. In support of these data we have observed replatable CFU-GM only in wells containing CA, and never in CA-negative wells that contained hematopoietic cells adherent to the stromal layer. Remarkably, in our micro-LTBMC all cells and CFU-G/M were associated with the adherent layer. This contrasts with ample reports on the shed of CFU-GM to the medium in flask cultures, and with an earlier study on the culture of human BMC in microtiter wells. Our observations therefore indicate that the measurement of CFU-G/M associated with the adherent layer gives information of the total well production of CFU-G/M.

The miniaturized (LTBMC) technique used here differs from previous studies with respect to the eradication of endogeneous hematopoietic activity. Micro-LTBMC have been reported earlier using 1.67 cm² surface area wells, but the use of 96-well plates has only once been reported for murine LTBMC. To ensure optimal survival and clonal amplification of any precursor cell inoculated a pre-existing stromal layer is required. This certainly does apply to fractionated BMC, in which the essential stromal cells are selectively depleted, but also to antineoplastic agent-treated BMC which might be inhibited in the establishment of the stroma, leading to loss of clonogenic hematopoietic cells. In our hands, eradication of endogeneous hematopoiesis by radiation of the established layer in flasks was highly reproducible. In contrast, we experienced either loss of stromal support for hematopoiesis or recurrent hematopoietic activity by lowering the horse serum and hydrocortisone concentrations in the culture media to free stromal layers of hematopoiesis according to the method of Reincke et al.12

It has been reported that 5-FU treatment of mice in vivo

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

**Fig 6.** Femoral content of CAFC (curve from Fig 3), CFU-GM, day 8 and day 12 CFU-S, and MRA at 3 days following injection with 150 mg 5-FU/kg BW. Arrows indicate the ranges within which depletion of the respective precursor cell pools in the marrow was induced by 5-FU treatment.

---

**Table 5. Average Half-Life of CA in Microcultures of Various BMC Fractions**

<table>
<thead>
<tr>
<th>Cell</th>
<th>No. of Experiments</th>
<th>Half-life of CA (d) (1 SE)</th>
<th>Observation Period (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated BMC</td>
<td>7</td>
<td>3.6 (0.1)</td>
<td>7-28</td>
</tr>
<tr>
<td>CCE+/Rh-bright</td>
<td>3</td>
<td>1.7 (0.1)</td>
<td>7-28</td>
</tr>
<tr>
<td>CCE/Rh-dull</td>
<td>3</td>
<td>6.0 (0.4)</td>
<td>9-28</td>
</tr>
<tr>
<td>Lin⁻/Rh-bright</td>
<td>3</td>
<td>1.5 (0.2)</td>
<td>7-28</td>
</tr>
<tr>
<td>Lin⁻/Rh-dull</td>
<td>3</td>
<td>17.2 (0.6)</td>
<td>9-28</td>
</tr>
<tr>
<td>5-FU 3d (150 mg/kg)</td>
<td>3</td>
<td>5.0 (0.1)</td>
<td>9-32</td>
</tr>
<tr>
<td>5-FU 3d (75 mg/kg)</td>
<td>3</td>
<td>4.0 (0.2)</td>
<td>9-32</td>
</tr>
</tbody>
</table>

*Sorted in the light scatter blast cell window from elutriated (CCE) or magnetic bead-treated (Lin⁻) BMC.*

---

IN VITRO ASSAY FOR HEMATOPOIETIC STEM CELLS 2761
leads to dramatic reduction of the number of erythroblasts, CFU-GM, and day-7 and day-13 CFU-S, but far less of MRA, in the bone marrow.\textsuperscript{24} Similarly, it appears from Fig 4 that the frequencies of cells forming CA between days 5 and 21 after overlay are decreasingly reduced in the marrow of 5-FU (150 mg/kg)-treated mice as compared with untreated mice. The micro-LTBMC assay using the limiting-dilution strategy therefore seems to offer an insight into the frequency distribution of the total stem cell hierarchy, ranging from the most mature progenitor cells as measured by the CAFC-3d frequencies to the most primitive hematopoietic stem cells (ie, MRA cells), detected by CA-formation after day 21. By virtue of their ability to generate new stem cells, the cells with MRA have long-term repopulating ability in vivo, and are of particular relevance to successful marrow transplantation. It appears from our study that the frequency of such cells in normal bone marrow, as determined by the presence of CA at day 28 or later, averages six per 10\textsuperscript{5} cells (see Table 3), or less. This is similar to the number of primitive stem cells in normal bone marrow estimated by limiting dilution calculation using (temporary) cure of the anemic W/W\textsuperscript{v} mice as an endpoint, and agrees reasonably well with the frequency of cells producing bone marrow clones in previously irradiated and subsequently repopulated mice.\textsuperscript{29}

A one-phase micro-LTBMC assay has recently been described for human cells.\textsuperscript{25} Provided sufficiently large human marrow biopsies, a two-phase assay can be developed using pre-established stromal layers. There exists no assay that quantitates human hematopoietic precursor cells equivalent to the murine stem cell assays for CFU-S and MRA. Since the quantitation of CFU-GM or CFU-Mix in a bone marrow transplant does not consistently relate to the extent and success of bone marrow transplantation (BMT),\textsuperscript{28} it is evident that a human assay for the enumeration of the frequency of long-term marrow-repopulating cells would be a unique tool in the qualification of an in vitro purged graft for autologous BMT purposes. It would also add a new dimension to the delineation of the defective stem-cell pool in aplastic anemia or leukemia, and to the quantification of hematopoietic toxicity accompanying cytoreductive therapy and BMT.

REFERENCES


7. Ploemacher RE, Brons NHC: Cells with marrow and spleen repopulating ability and forming spleen colonies on day 16, 12 and 8 are sequentially ordered on the basis of increasing rhodamine-123 retention. J Cell Physiol 136:531, 1988


10. Donowitz GR, Quesenberry PJ: 5-Fluorouracil effect on cultured murine stem cell progeny and peripheral leukocytes. Exp Hematol 14:207, 1986


An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse

RE Ploemacher, JP van der Sluijs, JS Voerman and NH Brons