Identification of a Light Density Murine Megakaryocyte Progenitor (LD-CFU-M)

By Christian Chatelain, Marc De Bast, and Michel Symann

Murine bone marrow cells were separated on discontinuous Percoll gradients and assayed for their ability to give rise to megakaryocyte colonies. Ninety-one percent of the megakaryocyte progenitors (CFU-M) sedimented at densities between 1.070 and 1.080 g/mL. Six percent of CFU-M were found at densities between 1.060 and 1.070 g/mL, 2% between 1.050 and 1.060 g/mL, whereas <1% had a density either lower than 1.050 g/mL or higher than 1.080 g/mL. The number of doublings and endomitoses achieved by progenitors of density classes higher than 1.050 g/mL were similar. However, colonies derived from CFU-M of densities <1.050 g/mL (LD-CFU-M) had a higher probability of polyploidization and a lower probability of cell division in vitro. The inverse correlation found between the number of cells per colony and their DNA content was invariable regardless of the density class of the progenitors. The heterogeneity of the ploidy of cells within colonies increased continuously with increasing cell numbers per colony. To study if a short-period exposure of LD-CFU-M to acute thrombocytopenia could modify the ploidy of their progeny, mice were given rabbit antmime platelet serum while control animals received normal rabbit serum. Twenty-four hours after injection, marrow was cultured. After a five-day culture period, no change in the number of colonies, doublings, ploidy, and heterogeneity of ploidy were observed between control and thrombocytopenic animals. The data suggest that LD-CFU-M are a distinct category of CFU-M, perhaps more mature than the common CFU-M.

PLATELET PRODUCTION depends on the proliferation and maturation of a hierarchy of progenitor cells. In mice, the earliest progenitor is a pluripotent cell defined by its ability to give rise to the erythroid, granulocytic, and megakaryocytic cell lineages, and to self-renew. Restriction of the differentiative capabilities of this stem cell to the megakaryocytic pathway leads to the production of a cell functionally designated the megakaryocyte colony forming unit (CFU-M). It is detectable only by in vitro culture techniques.

With common Romanowsky staining methods, the earliest morphologically recognizable platelet precursor is the 8N megakaryocyte. Cytochemistry permits the recognition of megakaryocytes at the diploid stage. The enzyme acetylcholinesterase (AchE) is limited to the megakaryocytic lineage in rodent bone marrow, although it has been found recently in some eosinophilic cells. Acute thrombocytopenia results in an increased proportion of small AchE-positive megakaryocytes, but it does not influence both number and ultimate ploidy of marrow CFU-M before changes are observed in marrow megakaryocytes and before the platelet count has recovered. However, the DNA content of megakaryocytes in big-cell-type colonies is increased four to five days after induction of acute thrombocytopenia.

Techniques for the separation of megakaryocyte progenitors according to their density properties have been reported earlier. Using Percoll, a medium consisting of polyvinylpyrrolidone-coated silica beads, Rabellino et al showed that human megakaryocytes sediment at densities <1.050 g/mL. Burstein et al have reported that murine megakaryocytes are found mainly at low densities while CFU-M are enriched in high-density fractions.

Recently, Ishibashi and Burstein separated murine megakaryocytes and their progenitors on continuous gradients of Percoll. Eighty-seven percent of megakaryocytes were found in fractions of density <1.058 g/mL and 86% of small AchE-positive cells were collected from fractions between 1.039 and 1.078 g/mL. No cells greater than 4N were found at densities higher than 1.071 g/mL. Most of the CFU-M appeared to sediment at about 1.074 g/mL.

To investigate differences in ploidy behavior of murine megakaryocyte progenitors, we studied their culture characteristics after separation on discontinuous Percoll gradients. When compared with the majority of CFU-M, light density-CFU-M have a higher probability of endomitosis and a more limited proliferative potential. However, following induction of short-term acute thrombocytopenia, light density-CFU-M behaved in an identical manner to the more common higher density progenitor.

MATERIALS AND METHODS

Mice. C57BL/6J mice (Jackson Laboratories, IFFA CREDO, Lyon, France), specific pathogen-free and 8 to 10 weeks old, were used for all experiments.

Preparation of antiplatelet serum. Rabbit antmime platelet serum was prepared as previously described. Blood drawn from the retoroorbital plexus two hours after intravenous (IV) injection of antiserum showed a platelet count of about 15% that of control values (control, \(1.177 \times 10^5/\mu L \pm 0.037;\) thrombocytopenia: \(0.186 \times 10^5/\mu L \pm 0.032\)). Although the degree of thrombocytopenia was not as severe as that induced by some investigators, the mean diameter of marrow megakaryocytes (800 in both groups) measured on smears was significantly increased 24 hours after induction of thrombocytopenia (37.8 \(\mu m \pm 1.4 \mu m\) and 42.7 \(\mu m \pm 3.0 \mu m\) in controls and thrombocytopenic mice, respectively; \(P < .001\)).

Percoll preparation. Basic Percoll solution was prepared by adding 1 vol of phosphate buffered saline (PBS) 10-fold (PBS3040; Eurobio, Paris) to 9 vol of Percoll (P1644, Sigma Chemical Co, St Louis, Missouri).

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Submitted February 26, 1987; accepted June 1, 1988.

Supported by Grant No. 3.4568.86 from The Fonds Belge de la Recherche Scientifique Médicale and The Caisse Générale d’Epargne et de Retraite.

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0006-4971/88/7204-0047$3.00/0

Louis). The solution was adjusted to a pH of 7.2 and passed through a 0.45-μm Millipore filter.

Each Percoll density was obtained using the following formula:

\[ A = \frac{1.123 - d}{d - 1.006} \]

where \( d \) represents the desired density and \( A \) represents the number of milliliters of PBS 1x to be added to 1 mL basic Percoll solution.

**Bone marrow separation.** To obtain various cell density fractions, murine bone marrow suspensions were separated on discontinuous Percoll gradients. Each layer consisted of 2 mL Percoll with densities ranging from 1.050 g/mL to 1.080 g/mL from the top to the bottom of a 15-mL plastic tube. Fractions differed by a density of 0.010 g/mL. The cell suspension was obtained by flushing femurs and tibiae with 3 mL of \( \alpha \)-medium (10-311-20; Flow Laboratories, Brauns), Cells were disrupted by successively passing them through 18-, 20-, and 23-gauge needles.

The cell suspension was layered at the top of the Percoll gradient and centrifuged at 250 g for 20 minutes at 4°C. The cells were harvested from each density interface, counted with a Neubauer hemocytometer after Trypan Blue dye exclusion, and cultured for CFU-M.

**CFU-M assay.** The number of megakaryocyte progenitors (CFU-M) in mouse bone marrow was measured after a five-day agar culture period following the method of Metcalf et al.2 modified by Burstein et al.4 Depending on the fractions, the number of cells plated varied between 20,000 and 165,000/plate. The number of colonies never exceeded 26/plate.

The pokeweed mitogen-stimulated spleen conditioned medium (PWM-SCM) was obtained by culturing mouse spleen cells in the presence of both 5% human plasma and pokeweed mitogen (5% of a 1:15 dilution; Gibco [Ghent, Belgium] 670-5360). This PWM-SCM was used as a source of megakaryocyte-colony stimulating activity (meg-CSA) and added to the CFU-M cultures at a final concentration of 20%, which gave optimal numbers of colonies.

**Megakaryocyte staining.** After a five-day culture period, the megakaryocyte colonies were enumerated and the agar disks were gently layered onto 50 x 75-mm glass slides, as previously described.4 The dried culture slides were stained for AChE for two hours by the method of Jackson.5

Since specificity of AChE stain has been questioned, Luxol fast blue stains were performed on whole culture slides.6 Although eosinophils were stained by Luxol fast blue on buffy-coat smears, no eosinophils were recognized within megakaryocyte colonies. Slides were then stained for 30 minutes using 1.75 x 10⁻⁵ mol/L Chromomycin A₃ (CA₃), washed, dried, and coverslipped with DPX mountant (BDH Chemicals, Richmond, CA).

**Ploidy measurements.** Fluorescence cytophotometric analysis of megakaryocytic ploidy in culture was performed using our recently published method.8 Briefly, megakaryocyte colonies were recognized by transmission microscopy. After an adequate fading period (<5% fading in 15 minutes), the fluorescence of megakaryocytes was measured using a Carl Zeiss III microscope (Carl Zeiss, Oberkochen, West Germany) equipped with an epifluorescence illuminator and a photomultiplier.

Granulocyte values served as the diploid reference standard. A computer (TRS-80 Model I; Radio Shack, Fort Worth, TX), interfaced with the photometer, was used for ploidy calculations. Data were stored on magnetic disks using programs written in BASIC for this purpose. The number of colonies per slide and the number of cells per colony were recorded.

**Statistics.** Results are expressed as the mean ± 1 SE. Statistical methods used were the two-tailed \( t \) test for two means, \( \chi^2 \) test, and linear regression analysis.

**RESULTS**

**Distribution of CFU-M.** Table 1 shows the distribution of CFU-M after separation on Percoll discontinuous gradients (fractions I to V). Most of CFU-M sedimented in a range from 1.070 to 1.080 g/mL. About 7% of CFU-M were found from 1.050 to 1.070 g/mL. Minimal numbers of megakaryocytic colonies were detected at densities either lower than 1.050 g/mL or higher than 1.080 g/mL. Cell recovery after Percoll separation was 72.7%, and megakaryocyte colony recovery was 52.5%. Daily examinations of cultures from day 0 to day 5 ruled out the possibility that mature megakaryocytes, possibly in clumps, can survive the separation processing and affect the number of colonies. Indeed, at day 2, no colony was ever observed.

**Doubling and endomitosis numbers achieved by CFU-M.** To study the proliferative capacity of CFU-M in a five-day culture period, the number of megakaryocytes per colony was determined. Figure 1 shows the distribution of colonies according to their number of doublings. The mean doubling numbers per colony calculated following our previously published criteria are 2.5, 4.0, 3.7, 3.6, and 3.5 for fractions I, II, III, IV, and V, respectively.8 There was no significant difference in the doubling numbers achieved by CFU-M from density fractions higher than 1.050 g/mL. However, CFU-M of a density lower than 1.050 g/mL have a decreased proliferative capacity, since their doubling num-

![Fig 1. Distributions of megakaryocyte numbers per colony (1. 2. 3. 4. 5. doublings = 2. 4. 8. . . cells, respectively) according to the density of progenitors. The bold lines represent the fraction of density <1.060 g/mL compared with other fractions (thin line). In this and subsequent figures, numbers of doublings refer to the logarithm base 2 of the cell number. All distributions are different from the <1.060 g/mL fraction by \( \chi^2 \) test \( (P < 0.001) \).](image-url)
LIGHT DENSITY MURINE MEGAKARYOCYTE PROGENITOR

Fig 2. Distributions of the geometric mean ploidy (number of endomitoses) per megakaryocytic colony according (1, 2, 3, ... endomitoses = 4N, 8N, 16N, ... respectively) to the density of progenitors. The bold lines represent the fraction of density <1.050 g/mL compared with other fractions (thin line). All distributions are different from the <1.050 g/mL fraction by \( P < 0.001 \).

Fig 3. Geometric mean ploidy of megakaryocytes per colony as a function of the number of doublings per colony. All correlations are linear (\( r = .96; P < .05 \)) and do not differ by a 95% confidence interval. (A), \(<1.050;\) (B), 1.050-1.060; (C), 1.060-1.070; (D), 1.070-1.080; (E), >1.080.

Fig 4. Ploidy heterogeneity (SD of the number of endomitoses per colony; Nb E) as a function of the number of doublings per colony. All correlations are linear (\( r = .81; P < .05 \)) and do not differ by a 95% confidence interval. (A), \(<1.050;\) (B), 1.050-1.060; (C), 1.060-1.070; (D), 1.070-1.080; (E), >1.080.

M-derived colonies did not differ from other CFU-M in this regard (within a 95% confidence interval).

The heterogeneity of ploidy within colonies is estimated by the SD of the number of endomitoses per colony. Figure 4 shows that ploidy heterogeneity increases linearly as the number of doublings increases. This linear relationship is the same regardless of the density fraction examined (within a 95% confidence interval).

Effect of acute thrombocytopenia on LD-CFU-M. To study the action of acute thrombocytopenia on LD-CFU-M, mice were rendered thrombocytopenic by IV injection of 0.2 mL antiplatelet serum while control mice received an equal amount of normal rabbit serum. Mice were killed 24 hours later. Table 2 shows the effect of acute thrombocytopenia on LD-CFU-M. Although the small numbers of marrow LD-CFU-M make comparisons difficult, they did not differ between the two groups of mice. Moreover, the number of doublings and the number of endomitoses per megakaryocytic colony were not significantly different in control and thrombocytopenic mice. There was no significant change in the number of doublings and the number of endomitoses achieved by LD-CFU-M, three and five days after induction of thrombocytopenia. No change in the linear correlations was observed when we considered endomitoses \( \nu \) doublings or heterogeneity \( \nu \) doublings achieved by LD-CFU-M in both groups (Figs 3 and 6).

DISCUSSION

Megakaryocytes are large polyploid cells, the physical properties of which have permitted their separation. There is some controversy in the literature concerning the estimated densities of mature megakaryocytes. Early studies performed with density centrifugation on bovine serum albumin concluded that mature megakaryocytes sediment at densities of about 1.10 g/mL.\(^{15}\) However, using Percoll continuous gradients, Ishibashi found that the majority of megakaryocytes have a density <1.058 g/mL.\(^{12}\) The reason for this discrepancy is unclear. A possible explanation refers to the impermeability of megakaryocytes to Percoll particles, making their density artificially lower.\(^{16}\) Raha et al compared...
density separation allows one to distinguish between progenitors and velocity sedimentation,\(^8\) and they sediment in Percoll in a range from 1.039 to 1.078 g/mL.\(^1\) Physical separation by density of the progenitors CFU-M has shown that these cells sediment in Percoll at a relatively high density, approximately 1.074 g/mL.\(^2\) Since Percoll density separation allows one to distinguish between progenitors (CFU-M) and mature megakaryocytes, it was concluded that measured density decreases as megakaryocyte maturation progresses.

This study was designed to focus on proliferation and endoreduplication of progenitors from various densities. No obvious differences were found among the various fractions of density >1.050 g/mL. However, CFU-M sedimenting in Percoll suspension of a density <1.050 g/mL attained about one doubling less than any other CFU-M. Since recognition criteria for colonies include that at least two cells are present, this progenitor is still able to divide at least one time, and therefore, is not yet involved in the polyplidization process. Indeed, polyploid megakaryocytes can no longer undergo cytoplasmic division. In our culture's conditions, about one extra endomitosis occurs in the progeny of this LD-CFU-M. Taken together, the number of divisions including mitoses and endomitoses is about the same for all progenitors in our culture, ie, six. However, in the case of LD-CFU-M, one doubling is replaced by one endomitosis, resulting in colonies of fewer cells with greater ploidy. These data support the idea that LD-CFU-M are more mature than other CFU-M and suggest that cell density decreases with progression of megakaryocyte maturation.\(^1\) The present work gives further insights into the distribution of colonies of different types. As reported earlier, the fluorescent method of ploidy measurement, having proved to be more accurate than the light transmission technique,\(^9\) shows again that a continuum of CFU-M exists with a progressive increase in endoreduplication potentiality. These data confirm the existence of different types of colonies as described by Lieschke and Levin.\(^19,20\) Moreover, intermediate colonies are constantly observed as previously published. Data reported by Paulus indicate also a correlation between ploidy levels and the number of cells per colony.\(^21\) Discrepancy with other previously published works that failed to suggest the correlation can be due to different culture conditions.\(^22,23\) The ploidy heterogeneity is clearly related to the number of endomitoses as demonstrated earlier for CFU-M.\(^2,8\)

One additional point we investigated was to examine the responsiveness of LD-CFU-M to a thrombocytopenic environment. Six to 24 hours after induction of acute thrombocyto-

### Table 2. Effect of Thrombocytopenia on LD-CFU-M

<table>
<thead>
<tr>
<th>Time After Antithrombocyte Serum Injection</th>
<th>No. of Doublings Per Colony</th>
<th>No. of Endomitoses Per Colony</th>
<th>LD-CFU-M Per Femur</th>
<th>Total No. of Experiments (Petri Dishes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 d Experimental</td>
<td>2.50 ± 0.07</td>
<td>2.37 ± 0.04</td>
<td>45.78 ± 0.54</td>
<td>4(76)</td>
</tr>
<tr>
<td>control</td>
<td>2.47 ± 0.09</td>
<td>2.39 ± 0.06</td>
<td>29.67 ± 6.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(P = .49)</td>
<td>(P = .49)</td>
<td>(P = .10)</td>
<td></td>
</tr>
<tr>
<td>3 d Experimental</td>
<td>2.05 ± 0.26</td>
<td>2.25 ± 0.09</td>
<td>15.59 ± 3.69</td>
<td>3(35)</td>
</tr>
<tr>
<td>control</td>
<td>2.00 ± 0.35</td>
<td>2.14 ± 0.26</td>
<td>22.99 ± 8.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(P = .46)</td>
<td>(P = .32)</td>
<td>(P = .17)</td>
<td></td>
</tr>
<tr>
<td>5 d Experimental</td>
<td>2.31 ± 0.34</td>
<td>2.34 ± 0.14</td>
<td>11.57 ± 3.29</td>
<td>3(35)</td>
</tr>
<tr>
<td>control</td>
<td>2.40 ± 0.51</td>
<td>2.32 ± 0.14</td>
<td>9.80 ± 3.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(P = .45)</td>
<td>(P = .47)</td>
<td>(P = .38)</td>
<td></td>
</tr>
</tbody>
</table>

Fig 6. Ploidy heterogeneity (SD of the number of endomitoses per colony; Nb E) as a function of the number of doublings per colony. The correlations are linear \((r \approx .96; P < .05)\) and do not differ by a 95% confidence interval.
topenia. After 24 hours, the volume and number of mature megakaryocytes increase while marrow CFU-M remain unchanged. When mice were rendered thrombocytopenic by injection of rabbit antiplatelet serum, the ploidy distribution, the colony size, and ploidy heterogeneity were identical to controls for the first 24 hours, whereas changes were observed four to five days later by Levin. Since LD-CFU-M account for a small proportion of CFU-M, any alteration in the numbers or characteristics of colonies derived from the low-density progenitors would not influence measurements made on the total progenitor pool.

In the present study, 24 hours after induction of acute thrombocytopenia, the numbers of LD-CFU-M per femur were not significantly different. In an attempt to determine the role of an acute thrombocytopenic environment on LD-CFU-M, characteristics of megakaryocyte colonies derived from LD-CFU-M were compared in normal and thrombocytopenic mice. The colony size and ploidy heterogeneity were identical to controls. This suggests that at a time when marked changes in megakaryocyte number and volume, and small AchE-positive cells occur, a similar short-term exposure to a thrombocytopenic environment has no direct effect on the properties of LD-CFU-M and, especially, on the ultimate ploidy achieved by the progeny of LD-CFU-M. The small trend in an increased number of doublings and endomitoses five days after induction of thrombocytopenia is not significant. The degree of thrombocytopenia reached in these experiments can be questioned. There are relationships between the severity of acute thrombocytopenia and the magnitude of the subsequent alterations in megakaryocytes. However, the platelet level achieved in the present study induced a significant augmentation in the mean marrow megakaryocyte size, proving its efficiency. Since LD-CFU-M seem to be less mature than small AchE-positive megakaryocytes, they appear to be progenitors functionally situated between the first progenitor committed to the megakaryocyte lineage (CFU-M) and the earliest morphologically recognizable megakaryocyte (the small AchE-positive megakaryocyte).

Since LD-CFU-M achieved more endomitoses than higher density CFU-M, the data are in agreement with the hypothesis of Paulus et al who isolated three classes of megakaryocyte progenitors with probabilities to endoreduplicate of 0.07, 0.48, and 0.74. Our data suggest that more mature CFU-M give rise to megakaryocytes of higher ploidy. The physiologic significance of LD-CFU-M could be soon revealed by the progressive appearance of purified growth factors and by the possibility of studying the timing of the expression of growth factor receptors.

ACKNOWLEDGMENT

The authors are indebted to Professor Gérard Sokal for his unyielding interest and support, to Dr S.A. Burstein (University of Oklahoma) for reviewing the manuscript, and to Cyndy Rozewicz and Gilda Manicourt for preparation of the manuscript.

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