Fluorescence Cytophotometric Analysis of Megakaryocytic Ploidy in Culture: Studies of Normal and Thrombocytopenic Mice

By Christian Chatelain and Samuel A. Burstein

A system for the accurate and rapid measurement of the ploidy of cultured megakaryocytes derived from megakaryocytic colony-forming cells (CFU-M) has been developed. Thirty thousand murine marrow cells per milliliter were cultured for varying time periods in agar in the presence of horse serum and pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM). To ensure the inclusion of all the megakaryocytic cells in the analysis, entire agar discs were transferred onto glass slides and dried. Cells of the megakaryocytic lineage were identified by staining for acetylcholinesterase (AchE) for two hours. Subsequently, the nuclei of the cells were stained using 1.7 x 10^-10 mol/L chromomycin A3, a specific DNA-binding fluorochrome. Megakaryocytic colonies (≥ 2 AchE + cells) were located under transmission light. The fluorescence emission of each cell of the colony was then measured by a photometer interfaced with a computer. The mean fluorescence emission of about 20 random granulocytes per slide was used as a 2N standard. There was no significant cell loss, quenching of fluorescence by AchE staining, or overlapping of colonies or cells. Approximately 100 megakaryocytes per hour could be analyzed. Modal ploidy of cultured megakaryocytes increased from 2N to 32N between days 3 and 6 in culture. Varying concentrations of PWM-SCM from 5% to 20% did not affect the ploidy distribution when examined at day 5. The heterogeneity of the ploidy of cells within colonies increased continuously with increasing cell numbers per colony. Clonal analyses of mean ploidy and ploidy heterogeneity did not show distinct types or classes of colonies; rather, the data show that megakaryocytic colonies are structured as a continuum. An inverse correlation was found between the number of cells constituting the colonies and their mean DNA content. To determine if short-term in vivo exposure of CFU-M to a thrombocytopenic environment could affect the ploidy of their progeny, mice were given rabbit antimouse-platelet serum while control animals were given normal rabbit serum. Twenty-four hours after injection, marrow derived from these animals was cultured. At day 5, the ploidy distributions and ploidy heterogeneity were identical in both treated and control groups. Thus, factor(s) that promote CFU-M proliferation do not affect megakaryocytic endoreduplication, while stimuli that acutely influence megakaryocytic ploidy in vivo do not determine the ultimate ploidy potential of megakaryocytes derived from a CFU-M.

In this article, we describe a method for measuring ploidy of cultured megakaryocytes that minimizes many of these limitations, and apply it to studies of normal and thrombocytopenic animals.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Me) specific pathogen-free and six to eight weeks old were used for all experiments.

Platelet Count

Blood was obtained from the retroorbital plexus. Platelet counts were determined employing a whole blood platelet counter on 10 µL of blood drawn into a diluent reservoir (Ultraflo, Clay-Adams, Parsippany, NJ).

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Preparation of Antiplatelet Serum

To induce thrombocytopenia, rabbit antimouse-platelet serum (APS) was prepared as previously described and injected intraperitoneally 24 hours before the animals were killed. Control mice received the same volume of normal rabbit serum (NRS). The platelet count two hours after APS was < 5% of control values.

Colony Assays

To obtain megakaryocytic and granulocyte-macrophage colonies, mouse femoral marrow cells were cultured in agar using a modification of the method of Metcalf et al. In the experiments involving administration of antiserum, marrow cells from the femurs were washed twice with 10 mL alpha medium before plating to remove contaminating plasma. To prevent overlapping of colonies, 30,000 marrow cells per milliliter were plated in all experiments. Pokeweed-mitogen-stimulated spleen cell-conditioned medium (PWM-SCM), a source of megakaryocytic colony-stimulating activity (meg-CSA), was added to cultures in concentrations of up to 20% of the culture volume. After varying times in culture, the colonies were enumerated according to previously defined criteria to ascertain that colonies would not be subsequently lost. Three milliliters of phosphate-buffered saline (PBS) was pipetted onto each plate, and the agar discs were rimmed with a spatula. After five minutes, the agar discs were gently poured onto 50 x 75-mm glass slides. To wash the agar discs, a sheet of filter paper (Whatman No. 1) was placed over one end of each plate, and removed when dry. Another sheet of filter paper and the process repeated (five times) until the agar discs were free of color (due to phenol red in the medium, and to the filter paper, and the process repeated (five times) until the agar discs were free of color (due to phenol red in the medium, and to the filter paper). An additional 2 mL of PBS were placed on the first sheet of filter paper, and the process repeated (five times) until the agar disc was free of color (due to phenol red in the medium, and to the serum). After careful removal of the filter paper, slides were allowed to dry and then fixed with acetone for five minutes.

Acetylcholinesterase (AchE) Staining

The dried culture slides were stained for AchE for two hours at room temperature by the method of Jackson. The slides were then rinsed in distilled water for one minute, and stained with chromomycin A3.

Chromomycin A3 (CA3) Staining

Ten milligrams of CA3, (-1.7 x 10^-5 mol/L), a 99% DNA-specific fluorochrome, was dissolved in 500 mL PBS, pH 8.0, supplemented with 10^-5 mol/L MgSO4. Slides were immersed in this solution in the dark at room temperature for 30 minutes. The slides were then rinsed in distilled water for one minute, and stained with chromomycin A3.

Ploidy Determination

Ploidy measurements were performed using a Zeiss III microscope equipped with an epifluorescence illuminator (250 W) and a photomultiplier (Carl Zeiss, Oberkochen, West Germany). To measure fluorescence emission, the epilluminator was turned on and a filter combination (Zeiss No. 48709) designed for measurement of fluorescein emission (the emission and excitation spectra of CA3 and fluorescein are similar) was switched into the light pathway. Under 500x oil immersion (Zeiss No. 518C), the circular field of measurement was restricted from 350 μm to 50 μm in diameter with an electromechanical shutter located between the epilluminator and the fluorescence filters. The field restriction as well as voltage, amplification gain, and time of measurement of the photomultiplier were controlled by a computer system (Zonax; Zeiss). To determine the diploid (2N) value, the fluorescence emission of granulocytes was measured. Granulocyte-macrophage colonies or clusters were present in all slides. At the periphery of such colonies, mouse granulocytes are often isolated and are easily recognized by their typical ring-shaped nuclear fluorescence. For each granulocyte, a nearby background measurement was performed. About 20 granulocytes were measured per slide.

Subsequently, the slide was scanned at 125x under transmission light to find AchE+ cells. Since megakaryocytes are generally observed as forming loose colonies in agar, it is probable that they have the capacity to migrate. Thus, to avoid the bias of measuring a cell that has drifted away from a colony, we did not measure isolated megakaryocytes. Only colonies containing two or more AchE+ cells were analyzed. However, some cells that were AchE+ but within the confines of a megakaryocytic colony were observed and their ploidy determined. The fluorescence measurements of megakaryocytes were performed exactly like those of granulocytes. Two background determinations were made per colony.

Calculations

Data were processed by the computer using a program written for this purpose and stored on floppy magnetic disks. The numbers of colonies per slide and the number of cells per colony were recorded. Differences between the value of each granulocyte (Gi) and its corresponding background (Bi) were obtained. The mean of these differences gave the mean diploid value. The fluorescence emission of each megakaryocyte (Mi) was then determined and the mean of the two corresponding background measurements (B1, B2) was subtracted. This number was then divided by the diploid value. The ploidy (MPi) was determined by multiplying this value by 2:

\[
MPi = \frac{Mi - B1 + B2}{2} \times \frac{2}{\left(\frac{G1}{B1}\right) - B2} \times 2
\]

where n is the number of measured granulocytes. Cells were assigned to ploidy classes by the method of Paulus et al. Statistical methods included linear regression and χ² analysis.

RESULTS

Time Requirements for Ploidy Measurements

Approximately one hour was necessary for removal of agar from eight plates, placement on slides, washing, drying, and fixation. Staining for AchE required two hours. Staining of the cells with CA3 required 30 minutes and fading of slides was completed in three hours. The time requirement for ploidy analysis was approximately two hours per slide (~ 100 megakaryocytes analyzed per hour). The majority of this time was spent searching for colonies (usually not in excess of 15 per slide).

Fluorescence Measurement

Fading of fluorescence emission is commonly observed with fluorescent dyes. The area of fading is limited to the area of illumination, ie, circles of 50 μm...
ANALYSIS OF PLOIDY IN CULTURE

Diameter during measurement and about 350 μm when centering the cells with an open-field stop. The initial rate of fading of CA3 was exponential, with a loss of intensity of up to 70% over the first hour. Thereafter, a relative plateau was achieved with a maximal fading rate of 5% over 30 minutes. Despite the initial rapid rate of fading, the fluorescence intensity remained extremely bright (Fig 1). Since all cells within colonies could be measured in less than 30 minutes irrespective of the size of the colony, variation in fluorescence intensity among colonies was less than 5% if measurements were carried out when fading had reached its plateau. Therefore, all slides were exposed to a 250 W incandescent bulb for three hours to ensure stability of fading. After a single three-hour exposure, daily measurements of the same faded granulocytes were similar for an observation period of ten days.

The basis of the histochemical stain for AchE is the precipitation of copper ferrocyanide at areas of enzyme activity. Since the brown heavy metal deposition could result in fluorescence quenching if staining times exceeded two hours, staining time was limited to two hours. When a smear of normal bone marrow was stained for AchE for two hours and the ploidy of stained cells measured, distinct ploidy classes were observed without overlap (data not shown). To ascertain that megakaryocytic colonies would not be missed using that staining time, slides were stained for two hours, the positive colonies enumerated, and then the slides were stained for an additional four hours. No additional colonies were identified following prolonged staining although some individual cells initially AchE became positive with the additional staining time.

**Determination of Diploid Value**

Granulocytes were present in all cultures and served as a diploid (2N) reference value. The fluorescence emission of each granulocyte was corrected by subtracting the corresponding background value. The fluorescence emission of granulocytes had a coefficient of variation of 15.6 ± 5.7% (mean ± SD from 32 slides; approximately 20 granulocytes per slide). Background fluorescence accounted for about 30% of the measurement of granulocytes and was found to be due mainly to the agar matrix. The glass and Diatex contributed to less than 5% of the fluorescence background.

**Megakaryocytic Ploidy**

The ploidy distribution of megakaryocytes within normal murine CFU-M-derived colonies measured after five days of culture has a mode of 16N (Fig 2). Minimal variation of this distribution was observed between experiments. Diploid cells were occasionally positive for AchE.

Figure 3 shows the geometric mean ploidy of cultured megakaryocytes as a function of the size of the colony following five days of culture. The larger the colony, the lower the mean ploidy of that colony. The colony size as well as the cellular DNA content increase by doubling. Therefore, colony size can be expressed as number of doublings (log2 of megakaryocyte number) and colony ploidy can be expressed as the mean and SD of the number of endoreduplications (log2 of ploidy - 1). An inverse linear correlation between cellular doubling and endoreduplication was found (r = .93; P < .05).

The standard deviation (SD) of the number of endoreduplications per colony was used to estimate the heterogeneity of ploidy of individual cells within colonies. As shown in Fig 4, at day 5 of culture, this SD increases linearly with colony size (r = .94; P < .05).

The distribution of ploidy on sequential days in culture is shown in Table 1. From days 3 to 6, the mode of the ploidy distribution increased daily reaching 32N.

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**Fig 1.** Megakaryocytic colony stained for AchE and DNA with CA3. (A) transmission light; (B) fluorescence. AchE staining for two hours does not quench the fluorescence emission of CA3.

**Fig 2.** Ploidy distribution of megakaryocytes at day 5 of culture. The bars represent the mean frequency (± 1 SE). Eighty-six colonies with a total of 821 cells in four experiments were counted for Figs 2, 3, and 4.
at day 6. Thereafter, dead or dying cells were evident as deteriorated nuclei often observed within megakaryocytic colonies, resulting in progressive uncertainty of measurement.

The effect of varying concentrations of PWM-SCM on ploidy distribution of day-5 colonies is presented in Table 2. PWM-SCM concentrations ranging from 5.0% to 20% resulted in the same overall ploidy distribution, although an increase in both the number of colonies and the number of cells per colony was observed.

The lack of influence of thrombocytopenia on the ultimate ploidy of CFU-M-derived cells is shown in Fig 5. Mice were made thrombocytopenic with APS while controls were given NRS. Twenty-four hours later, the marrow was removed, washed twice to remove contaminating plasma, and set up in culture. At day 5, ploidy analysis of the resultant CFU-M-derived colonies was performed. The general ploidy distribution (Fig 5), the correlation between the number of cells per colony and mean ploidy per colony (Fig 6) and the correlation between ploidy heterogeneity within colonies and the number of cells per colony (Fig 7) were identical in cultures derived from thrombocytopenic and control mice.

DISCUSSION

The capability to grow megakaryocytic progenitor cells in vitro has made it possible to examine the events that affect early megakaryocytopoiesis. It has become apparent through such studies that megakaryocytopoiesis is regulated on at least two levels.18,24-26 Early megakaryocytopoiesis, consisting of the clonal expansion of CFU-M, responds to factor(s) derived from mitogen-stimulated spleen cells or the supernatants of various cell lines. Late megakaryocytopoiesis, consisting of polyploidization and cytoplasmatic maturation, responds in vivo to perturbation of the platelet count and in vitro to plasma extracts and the supernatants of various cultured cells and cell lines.14,16,27-29 To study the potential relationships between CFU-M and megakaryocytic ploidy with the goal of learning more about the control mechanisms of these events, we set out to measure directly the DNA content of cultured megakaryocytes. In this endeavor it was important that (1) no cells were lost in the preparation of the specimens to be examined; (2) colonies would not overlap, ensuring that each cell in a colony was a member of the clone; (3) the megakaryocytic nature of the colony be documented by some independent marker; and (4) the method was rapid, stable, precise, and relatively inexpensive.

To obviate colony overlap, we chose to plate 30,000 cells per milliliter of culture, resulting in a maximum of 15 colonies per dish. To avoid cell loss and selection bias associated with plucking of colonies, the entire agar culture was removed from the dishes and dried down on glass slides. Enumeration of colonies before removal and after AchE staining showed that no colonies were lost. The process of drying down the agar discs resulted in the additional benefit of a slight degree of spreading of cells within colonies. Thus, it was rare to observe overlapping of individual megakaryocytes within a colony. The capacity to stain the cultures for both AchE and DNA in a permanent preparation permitted independent identification of the colonies, and shortened the time necessary for colony localization.

The use of fluorescence emission for the determination of DNA has several advantages over that of absorbance cytofluorometry.17 Fluorescent molecules

![Fig 3](image3.png) Geometric mean ploidy of megakaryocytes per colony as a function of the number of cells per colony. The correlation is linear \((r = .93; P < .05)\).

![Fig 4](image4.png) Ploidy heterogeneity (SD of the number of endoreduplications per colony; NbE) as a function of the number \((\log_2)\) of cells per colony (doublings). The correlation is linear \((r = .94; P < .05)\).

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**Table 1. Ploidy Distributions at Sequential Days of Culture**

<table>
<thead>
<tr>
<th>Day of Culture</th>
<th>Ploidy Class</th>
<th>Mean No. of Cells per Colony</th>
<th>No. of Colonies Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2N 4N 8N 16N 32N 64N</td>
<td>5</td>
<td>34†</td>
</tr>
<tr>
<td>4</td>
<td>11 21 19 32 15 2</td>
<td>7</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>10 12 22 38 16 2</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>6</td>
<td>7 10 20 23 36 4</td>
<td>11</td>
<td>39</td>
</tr>
</tbody>
</table>

*Percentage of total cells in each ploidy class. Cultures stimulated with 20% PWM-SCM. The modal ploidy at each day is in italics. †1,930 cells were counted for this experiment.
emitted light in all directions. Light quanta entering the front lens of an objective produce the same photometer reading whether the structures emitting the light are evenly or irregularly distributed in the measuring field.

With absorbance photometry, irregular distribution of DNA within a nucleus requires that an integration be performed of the extinctions of individual points at different sites in the nucleus, generally requiring expensive densitometry apparatus. By measuring fluorescence, a single reading of the entire nucleus is a direct measure of the total amount of dye bound by the DNA. The only equipment required is a microscope that can be adapted for epillumination, fluorescence filters, and a photometer. Although we used a computer interface with the microscope to facilitate shutter manipulations and automatically record and calculate the data, these procedures can be done manually without a computer.

Chromomycin A3 was chosen for these studies for several reasons. CA3 was first used for the identification of megakaryocytic ploidy in flow cytometry. It is an intense and specific DNA-binding fluorochrome that will react with fixed cells within minutes. Fluorochromes which have been generally used for fluorescence determination of DNA, such as propidium iodide, have the disadvantage of also binding to RNA. Preparations prepared with the latter fluorochrome thus require prior treatment with RNAase.

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The present data demonstrate that the modal ploidy increased over time in culture, as previously described. Because of nuclear deterioration in some cells beginning after day 6, we did not measure later time points. However, increasing concentrations of PWM-SCM did not affect the ploidy distribution when measured at day 5, despite increasing the total number of colonies and the mean number of cells per colony. This suggests that at the concentrations tested, meg-CSA present in this conditioned medium does not directly affect late megakaryocytopoiesis.

We and others have reported that megakaryocytic colonies are heterogeneous in appearance. Employing the methodology described, a colony-by-colony analysis was performed to quantitate the heterogeneity of colonies. If a colony contains three cells, each of 16N ploidy, the mean number of endoreplications of the colony is three and the SD of the mean is zero. Conversely, if a colony consists of three cells of 8N, 16N, and 32N, the mean number of endoreplications is three with a SD of one. Thus, an estimate of the ploidy heterogeneity of a colony is given by the SD of the number of endoreplications of that colony: the more heterogeneous, the higher the SD. When the relationship between the number of cells per

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### Table 2. Effect of meg-CSA on Ploidy Distribution

<table>
<thead>
<tr>
<th>Ploidy Class</th>
<th>No. of Colonies/3 x 10^4</th>
<th>Mean No. of Cells per Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>2N</td>
<td>41</td>
<td>7</td>
</tr>
<tr>
<td>4N</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>8N</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>16N</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>32N</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>64N</td>
<td>21</td>
<td>2</td>
</tr>
</tbody>
</table>

*Percentage of total cells in each ploidy class.
†Distributions observed with 5%, 10%, 15%, and 20% PWM-SCM were not different by χ² test (P > .05).
colonies and the mean ploidy of that colony was examined, an inverse linear relationship was found. Although the slopes of the regression lines represented in Figure 3 (normal mice) and Figure 6 appear different, the 95% confidence belts overlap over the full range of these experiments. Changes in culture conditions may account for variation in slopes. A similar inverse relationship between number of endoreduplications and number of doublings was noted by others using a plasma clot system supplemented with erythropoietin. However, this relationship was not observed by others using culture conditions similar to ours. When the SD of the number of endoreduplications of each colony was examined with respect to the numbers of cells in that colony, a direct linear relationship was observed. These results emphasize that visual observation of colonies in situ can be misleading. Megakaryocytic colonies do not fit into defined categories; rather, a continuum of colony size and ploidy heterogeneity exists.

To determine the influence of late regulatory factor(s) on the ultimate ploidy achieved by the progeny of CFU-M, mice were made thrombocytopenic with APS, while the control animals received NRS. Twenty-four hours later the animals were killed and the marrow cultured. This time point was chosen because of previous in vitro studies showing that CFU-Ms do not increase despite an increase in megakaryocyte size when examined 24 hours after injection of APS. The ploidy distributions, colony size, and ploidy heterogeneity were identical to controls. Since colonies were measured only on day 5, it is possible that a major increase in modal ploidy could have occurred at an earlier time point such that by day 5 the high ploidy cells would have disintegrated. This is quite unlikely however, since dying cells would have been easily recognized. These data suggest that an acute thrombocytopenic environment does not influence the ultimate ploidy of the progeny of CFU-M. A similar result was described by Levin et al. when ploidy distributions were analyzed following two days of thrombocytopenia.

Our data that colony size and ploidy heterogeneity are a continuum does not contradict the conclusions of others that megakaryocytic colonies arise from different types of progenitors. It is possible, and perhaps probable, that there is a hierarchy of CFU-M. None of these progenitors may be directly responsive to late regulatory factors, but the closer the progenitor is in its state of differentiation to the committed 2N megakaryocyte, the greater the probability it may have to leave the duplication pathway and enter the pathway of polyploidization.

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