Interrelationship Between Mitosis and Endomitosis in Cultures of Human Megakaryocyte Progenitor Cells

By Moises Arriaga, Karen South, Janet Lee Cohen, and Eric M. Mazur

Sera from dogs rendered aplastic by total-body irradiation stimulate human bone marrow megakaryocyte progenitors to form megakaryocyte colonies in plasma clot cultures. In this investigation, we evaluated the effects of varying concentrations of such sera on both the mitotic and endomitotic phases of human megakaryocyte development in vitro. When low concentrations of aplastic canine sera (2.5% to 5.0% [vol/vol]) were added to cultures of human peripheral blood mononuclear cells in place of normal AB serum, megakaryocyte colony formation was augmented fivefold, cell numbers per colony increased approximately 2.5-fold, and the geometric mean megakaryocyte ploidy almost doubled. Further increasing the aplastic canine serum concentration from 10% to 30% (vol/vol) stimulated no additional colony formation. However, there was a further augmentation of cell numbers per colony associated with a progressive decrease in the mean megakaryocyte ploidy. Megakaryocyte cultures were harvested after 7, 12, 15, and 19 days of incubation, and these demonstrated that the lower mean ploidy values found at the higher concentrations of aplastic canine serum did not result from delayed endoreduplication. At all aplastic serum concentrations evaluated, there existed a strong correlation between nuclear ploidy and cell diameter. We conclude that both the mitotic and endomitotic events in human megakaryocytopenia may be influenced by a factor or factors present in aplastic canine serum. At lower in vitro concentrations, such sera stimulate both mitosis and endomitosis, which promotes the development of megakaryocyte colonies composed of larger cells with a higher mean ploidy. With increasing aplastic serum concentrations, colony formation plateaus and mitosis is favored over endomitosis. This results in colonies composed of more numerous but smaller megakaryocytes with a lower mean ploidy. Our data suggest that the size and extent of polyploidy that can be achieved by a developing megakaryocyte may be influenced by the mitotic prior history of its immediate precursor cell.

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MATERIALS AND METHODS

Human subjects. Peripheral blood for CFU-Meg cultures and normal AB sera was obtained by routine venipuncture from consenting healthy adult donors who provided written informed consent.

Canine sera. Aplastic canine sera were prepared as previously described. The lot of serum used in this investigation was harvested from an animal whose platelet count was 4,000/μL at the time of sacrifice.

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**Plasm clot megakaryocyte cultures.** Human peripheral blood was diluted 1:1 with α-medium minus nucleosides (GIBCO, Grand Island, NY) containing preservative-free heparin at 20 U/mL. Mononuclear cells were isolated by Ficoll 400 (density 1.077 g/mL) (Lampire Biologicals, Pottstown, PA) centrifugation of the cells were suspended in α-medium containing 20% AB serum, and the plastic-adherent cells were removed by a one-hour incubation at 37 °C in 100% humidified atmosphere of CO₂. In our laboratory, Feulgen/BAO staining is 96% DNA quantitated by DNA fluorochrome because its relative stability to UV oxidation (I), (BAO; Fluka Chemical Corp. Happauge, NY) was completely scanned, and rhodamine-positive colonies enumerated by fluorescence was reduced from 49.2 to 2.0 (arbitrary units).

**Immunofluorescent staining and scoring of cultures.** Rabbit anti serum to platelet glycoproteins (PGP) was prepared and its specificity demonstrated as previously reported.23 Megakaryocyte colonies were labeled immunofluorescently by sequential incubation with PGP antiserum diluted 1:200 in PBS and rhodamine-conjugated goat antirabbit IgG (IgG fraction) diluted 1:60 in PBS (Cappel Laboratories, Cochranville, PA). A rhodamine rather than fluorescein conjugate was used for the second label because its optical signal was stable to the acid hydrolysis required for DNA quantitation. In our laboratory, Feulgen/BAO staining is 96% DNA specific based upon the reduction in the nuclear fluorescence signal induced by exposure to deoxyribonuclease I (Sigma Chemical Co, St Louis) prior to staining. For this determination, human peripheral blood smears were fixed in methanol:acetone (1:1), and the test smears were preincubated in DNAase I at 0.5 mg/mL in 0.03 mol/L magnesium sulfate, pH 6.0. Control and test smears were Feulgen stained with BAO as will be described and the mean nuclear fluorescence was reduced from 49.2 to 2.0 (arbitrary units).

The fixed, PGP-rhodamine-labeled plasma clot cultures were rehydrated with distilled water, and then the DNA was hydrolyzed in 4 N HCl at 37 °C for 20 minutes. Hydrolysis was terminated by aspirating the HCI and flooding the culture dishes with distilled water at room temperature. Nuclear DNA was stained using freshly prepared BAO reagent (20 parts 0.01% BAO in distilled water [wt/vol] to two parts 1 N HCl to one part 10% sodium metabisulfite [wt/vol]). One milliliter of BAO reagent was layered on each 35-mm Petri dish, and the cultures were gently agitated for 30 minutes at room temperature in a 100% humidified chamber. BAO staining was followed by three two-minute washes with distilled water (18 parts distilled water, one part 1 N HCl, one part 10% sodium metabisulfite [wt/vol]), one ten-minute wash with distilled water, and dehydration with 70% ethanol. The culture dishes were air-dried, the plastic sides removed, and circular coverslips mounted with glycerol.

Megakaryocyte DNA quantitation was performed using a Leitz Laborlux 12 microscope equipped with an adjustable measuring diaphragm, an MPV compact microscope photometer, and interchangeable filter cubes (Ernst Leitz, Wetzlar, W Germany) interfaced with an Epson HX-20 computer (Epson America, Inc, Torrance, CA). The measuring diaphragm was located in the emitted light pathway, and adjustment of its aperture did not influence incident illumination.4 Green-band "Cube N2" (excitation filter BP 530 to 560 nm and barrier filter LP 580 nm) was used for the identification and enumeration of the PGP-rhodamine-labeled megakaryocytes, and wide-band UV "Cube A" (excitation filter BP 340 to 380 nm and barrier filter LP 430 nm) was used to measure BAO fluorescence.

At a magnification of 100× the megakaryocyte colonies were identified in situ in the plasma clot by rhodamine fluorescence, and single megakaryocytes were randomly selected for DNA quantitation. When the total number of megakaryocytes in a culture plate exceeded 200, representative sampling was assured by (1) measuring nuclear DNA in equal numbers of cells from both halves of that culture plate and (2) analyzing no more than three to four arbitrarily selected megakaryocytes in each colony. Within the colonies, cells were chosen based upon the order in which they were encountered while scanning using green incident light at 400×. This sampling algorithm was validated in ten representative instances by comparing repeat ploidy distributions determined each from 100 randomly chosen megakaryocytes in the same culture plate. Ploidy distributions did not differ significantly between paired determinations, and duplicate mean ploidy values had coefficients of variation averaging 8.6% ± 5.9% (±SD).

Megakaryocyte DNA was measured using UV light excitation. At a magnification of 400× the nucleus of each megakaryocyte was isolated using the adjustable measuring diaphragm, and its BAO fluorescent signal was quantitated photometrically. The background readings were taken in the same high-power field as each megakaryocyte using the same diaphragm aperture. The net megakaryocyte DNA emission signal (Mₙ) was calculated as the difference of the two values. Readings of diploid-cell DNA fluorescence, Dₙ (corrected for the background) were obtained throughout the culture specimen in a diploid cell:megakaryocyte ratio of approximately 1:2. When a culture dish was completely scored, the relative DNA content per cell for each of the megakaryocytes was determined using the following relationship:

\[
\text{Megakaryocyte DNA content (i)} = \frac{M}{\sum \frac{c_i D_i}{2}}
\]

where n was the number of diploid cells evaluated in that culture dish. Megakaryocytes were assigned to the ploidy level of the nearest modal value of DNA content according to the algorithm of Paulus et al.18 The measurements, calculations, and ploidy assignment were performed.
performed using an on-line Epson HX-20 computer that permitted the evaluation of 75 to 100 cells per hour. Each high-power field received a total of no more than 30 seconds of UV light exposure.

The mean megakaryocyte ploidy was calculated from the following equation:

$$\text{Mean megakaryocyte ploidy} = \text{anti-log} \left( \frac{\sum_{p=2}^{64} \log_2(p) \times n_p}{n} \right)$$

where $n_p$ is the number of megakaryocytes in ploidy class $p$ ($p = 2, 4, 8, \ldots, 64$) and $n$ is the combined number of megakaryocytes in all ploidy classes. The geometric mean megakaryocyte ploidy is a more accurate representation of the mean ploidy than the linear average used in some studies because the linear average disproportionately weights the contribution of higher-ploidy megakaryocytes.

**Megakaryocyte size.** Megakaryocyte diameters were determined at 400× magnification using a calibrated eyepiece micrometer.

**Statistical analyses.** Megakaryocyte ploidy distributions were compared by Pearson's $\chi^2$ test of independence of distributions. Other comparisons were made using Student's $t$ test or linear regression analyses.

**RESULTS**

Similar to what we have reported previously,8 low-level megakaryocyte colony formation from peripheral blood mononuclear cells was observed in unstimulated cultures containing only normal human AB serum. In such cultures 10.4 ± 3.8 (mean ± SEM) megakaryocyte colonies were grown from 10⁶ mononuclear cells (Fig 1). The addition of aplastic canine serum stimulated up to a fivefold increase in colony formation (Fig 1). The megakaryocyte colony numbers reached a maximum of 54.4 ± 11.2 (mean ± SEM) at an aplastic canine serum concentration in a culture of 2.5% (vol/vol). With further increases in the aplastic canine serum concentration, the megakaryocyte colony formation plateaued (Fig 1).

Aplastic canine serum also enhanced the number of cells developing in each megakaryocyte colony, but there was no plateau in the dose-response effect. The mean number of megakaryocytes in each colony increased almost monotonically throughout the entire range of aplastic canine serum evaluated (Fig 1). Cells per colony rose from 5.8 ± 0.5 (mean ± SEM) in cultures containing only normal AB serum to 46.1 ± 11.4 (mean ± SEM) at the maximum aplastic canine serum concentration of 30% (vol/vol). The distribution of megakaryocyte colony size as a function of aplastic canine serum concentration is presented in Table 1. With increasing aplastic canine serum concentrations, there was a continuous upward shift in the modal colony size. In addition, the maximal colony sizes progressively increased, which reflected the stimulation of up to four mitotic generations beyond that observed in unstimulated cultures.

The same cultures used to evaluate colony numbers and size were stained with BAO for DNA quantitation. Because individual megakaryocyte colonies grown in plasma clot cultures are typically dispersed,14 overlapping of constituent cells was not a significant problem in performing such measurements. Illustrated in Fig 2 is a representative megakaryocyte colony in which both megakaryocyte cytoplasm is labeled immunochemically with anti-CD34/CD41 antibody and nuclei are stained with BAO. In Fig 2B there are additional cells whose nuclei stain with BAO but do not label with the anti-CD34/CD41 antibody. These cells, consisting predominantly of macrophages and lymphocytes, served as the diploid standards from which megakaryocyte ploidy was calculated. The coefficient of variation for the diploid cell DNA fluorescent signals averaged 32.5%, and the background fluorescence constituted approximately 30% of the total 2N nuclear measurements.

The effect of increasing concentrations of aplastic canine serum on cultured megakaryocyte mean ploidy was biphasic (Fig 3). Between aplastic serum concentrations of 0% and 2.5% (vol/vol), a significant enhancement of ploidy was observed, with the mean values rising from 9.67N at 0% to 12.66N at 2.5% ($P < .01$ by Pearson's $\chi^2$). The peak megakaryocyte ploidy was observed at the same aplastic serum concentration as that at which colony formation attained its maximum (Fig 1). The mean ploidy decreased with further increases in the aplastic serum concentration and reached a minimum of 4.98N at 30% aplastic serum. The enhancement of megakaryocyte ploidy by aplastic serum at concentrations of 2.5% to 10% was a result of a shift in the distribution of cells from the 4N and 8N to the 16N and 32N ploidy classes. At aplastic canine serum concentrations of 15% to 30% in culture, a decreasing mean ploidy resulted from increases in 2N and 4N cells at the expense of 16N, 32N, and 64N cells (Table 2).

The kinetic aspects of megakaryocyte polyploidization in culture were determined to evaluate the impact of culture duration on ploidy. Megakaryocyte cultures containing 0%, 2.5%, and 20% aplastic canine serum were harvested serially following 7, 12, 15, and 19 days of incubation (Fig 4). The peak mean ploidy values were observed on days 12 to 15 of...
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Table 1. Distribution of Megakaryocyte Colony Size as a Function of the Concentration of Aplastic Canine Serum in Culture

<table>
<thead>
<tr>
<th>Aplastic Canine Serum Concentration (%)</th>
<th>No. of Megakaryocyte Colonies Evaluated</th>
<th>Mean Number of Mitotic Generations† (Megakaryocytes/Colony - Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>63.3, 23.3, 13.3, 0, 0, 0, 0</td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>29.6, 33.3, 27.8, 9.3, 0, 0</td>
</tr>
<tr>
<td>2.5</td>
<td>143</td>
<td>29.4, 28.0, 26.6, 14.7, 1.4, 0</td>
</tr>
<tr>
<td>5</td>
<td>140</td>
<td>12.1, 37.1, 34.3, 13.6, 2.9, 0</td>
</tr>
<tr>
<td>10</td>
<td>147</td>
<td>8.2, 27.2, 42.9, 17.7, 4.1, 0</td>
</tr>
<tr>
<td>15</td>
<td>107</td>
<td>7.5, 15.0, 32.7, 28.0, 9.4, 7.5</td>
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<td>20</td>
<td>155</td>
<td>8.4, 24.5, 32.3, 23.9, 10.3, 0.7</td>
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<tr>
<td>30</td>
<td>90</td>
<td>7.8, 13.3, 25.6, 25.6, 11.1, 10.0</td>
</tr>
</tbody>
</table>

†The interval range for colony size is calculated as $2^{n-0.5}$ to $2^{n+0.5}$, where $n$ represents successive mitotic generations (doublings). The values are given as the percentage of the total colonies.

The data are derived from three experiments and a total of 866 colonies. The arithmetic means and statistical analysis of these data are presented in Fig 1.

The concentration (vol/vol) of aplastic canine serum in culture replacing an equal volume of normal AB serum.

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The values are given as the percentage of the total colonies.

culture regardless of the concentration of aplastic serum used. The biphasic relationship of mean ploidy with aplastic serum concentration was seen as early as seven days and persisted through day 15 of culture. By day 19, many of the megakaryocyte colonies had degenerated, and the mean ploidies became equivalent at all serum concentrations tested. In the cultures containing 20% aplastic canine serum, there was no increase in ploidy beyond 12 days of culture. Thus, the lower mean ploidy seen in day 12 cultures (Fig 3) was not simply the result of delayed endoreduplication.

Because megakaryocyte size is known to vary with ploidy in vivo,4 this relationship was analyzed in the in vitro culture system. At all aplastic canine serum concentrations evaluated, the megakaryocyte size varied significantly with log$_3$ (ploidy). The slopes and intercepts of the regression lines relating these two parameters did not vary significantly among the different aplastic serum concentrations (data not shown). Therefore, megakaryocyte size vs ploidy data from all aplastic canine serum concentrations were consolidated and are presented in Fig 5.

DISCUSSION

The production of platelets from the committed megakaryocyte progenitor cell or CFU-Meg is complex, requiring nearly sequential mitosis, endomitosis, and cytoplasmic maturation.7 However, the physiological regulatory control systems governing these processes are for the most part poorly understood. We have recently reported that sera from patients with bone marrow megakaryocyte aplasia1,2 and from dogs with irradiation-induced aplasia3 contain a substance, Meg-CSF, that stimulates mitosis and the early development of human bone marrow CFU-Meg. Meg-CSF manifests this effect in culture by augmenting both the number of megakaryocyte colonies grown and the number of component cells developing in each colony.1,3

In this report, we establish that Meg-CSF containing aplastic canine serum has qualitatively similar effects on human peripheral blood CFU-Meg progenitors. We have also been able to demonstrate an interaction between varying mitotic stimulation of early megakaryocytopoiesis by aplastic serum and the degree of megakaryocyte polyploidization achieved in culture.

We observed an unexpected biphasic effect of increasing concentrations of aplastic canine serum on in vitro megakaryocytopoiesis. Compared with control cultures containing only normal AB serum, low culture concentrations of aplastic canine serum (2.5% to 5.0% [vol/vol]) augmented megakaryocyte colony formation fivefold, increased cell numbers

![Fig 2. Representative megakaryocyte colony double labeled with PGP antiserum/rhodamine (A, green incident light) and BAO (B, UV incident light) that was cultured from human peripheral blood mononuclear cells in the presence of aplastic canine serum (original magnification x 100).](www.bloodjournal.org)
per colony approximately 2.5-fold, and almost doubled the mean ploidy. Further increasing the aplastic serum concentration from 10% to 30% (vol/vol) stimulated no additional colony formation. However, there was a further augmentation of cell numbers per colony associated with a progressive decrease in the mean megakaryocyte ploidy. Thus, in those cultures containing the higher concentrations of aplastic canine serum, an inverse relationship was present between mitotic amplification of the megakaryocyte progenitor (as manifested by cell numbers per colony) and the extent of megakaryocyte endoreduplication.

Our results appear to conflict with similar studies in mice reported by Chatelain and Burstein. They found no effect of increasing concentrations of pokeweed mitogen–stimulated spleen conditioned medium on cultured murine megakaryocyte ploidy, despite slight augmentations in colony numbers and size. The effects that we observed using aplastic canine serum may therefore be dependent upon this specific source of Meg-CSF, result from the higher cell plating densities we used, or reflect the substantially greater changes in colony size seen in our studies.

That megakaryocyte development from its progenitor may be characterized by a reciprocal relationship between mitosis and endomitosis has been demonstrated previously by other investigators. However, these earlier studies analyzed differences among individual murine megakaryocyte colonies that were generally cultured under conditions providing maximal growth stimulation. It was found that colonies with larger numbers of cells had lower mean ploidy values. Cell number and mean ploidy differences among colonies were attributed to an intrinsic heterogeneity of the CFU-Meg progenitor cell population.

Our studies indicate that, although CFU-Meg heterogeneity may exist, mitosis and endomitosis can be further influenced in culture by a factor or factors present in aplastic canine serum. Because colony numbers plateau at aplastic serum concentrations of 2.5% and above, it is probable that the higher aplastic serum concentrations affect a fixed population of megakaryocyte progenitors rather than selectively recruit different progenitor classes. Therefore, the

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**Table 2. Ploidy Distributions of Progenitor Cell—Derived Megakaryocytes Cultured With Varying Concentrations of Aplastic Canine Serum**

<table>
<thead>
<tr>
<th>Aplastic Canine Serum Concentration (%)</th>
<th>No. of Megakaryocytes Evaluated</th>
<th>Ploidy Class of Megakaryocytes</th>
<th>2N</th>
<th>4N</th>
<th>8N</th>
<th>16N</th>
<th>32N</th>
<th>64N</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>111</td>
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<td></td>
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<td></td>
</tr>
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<td>1</td>
<td>92</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>2.5</td>
<td>275</td>
<td></td>
<td>4.5</td>
<td>27.0</td>
<td>35.1</td>
<td>20.7</td>
<td>7.2</td>
<td>5.4</td>
</tr>
<tr>
<td>5</td>
<td>375</td>
<td></td>
<td>7.6</td>
<td>22.8</td>
<td>46.7</td>
<td>15.2</td>
<td>7.6</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>348†</td>
<td></td>
<td>4.7</td>
<td>13.8</td>
<td>24.4</td>
<td>31.6</td>
<td>18.5</td>
<td>6.9</td>
</tr>
<tr>
<td>15</td>
<td>364</td>
<td></td>
<td>6.4</td>
<td>12.8</td>
<td>28.8</td>
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<td>190</td>
<td></td>
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<td>12.7</td>
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<td>27.4</td>
<td>11.6</td>
<td>2.6</td>
<td>0.5</td>
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</table>

The data represent three experiments and a total of 2,078 megakaryocytes. The geometric means and statistical analyses of these data are presented in Fig 3.

*The concentration (vol/vol) of aplastic canine serum in culture replacing an equal volume of normal AB serum.
†The values are given as a percentage of the total cells.
‡One cell (0.3% of total) classified as 128N.
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inverse relationship between mitosis and endomitosis that was previously attributed exclusively to progenitor cell heterogeneity likely also characterizes megakaryocytopoiesis in general. It is speculative whether this relationship is physiologically adaptive and operates in vivo. However, one may hypothesize that in conditions of severe megakaryocytic hypoplasia with accompanying thrombocytopenia, mitotic amplification of existing megakaryocyte precursors, even at the expense of endomitosis, may most effectively restore platelet production. This hypothesis is supported by a recent report by Tanum who demonstrated that the resumption of platelet synthesis in rats receiving a sublethal dose of thio-TEPA is temporally associated with the exclusive appearance of low-ploidy megakaryocytes in the bone marrow.18

Although Meg-CSF is known to be present in aplastic canine sera, this investigation does not distinguish between its effects and those of other regulators potentially present in such sera. The existence of additional regulators that influence megakaryocyte endoreduplication and cytoplasmic maturation have been postulated by several investigators.19-22 However, even if these exist, our data indicate that their influence may be limited by the mitotic history of the cells upon which they act. Another hypothesis consistent with our data is that Meg-CSF alone controls both the mitotic and endomitotic phases of megakaryocytopoiesis, with differential effects depending upon the intensity of Meg-CSF stimulation.

The normal AB sera used in our experiments may also have included factors capable of affecting megakaryocyte development in vitro. Several investigators have suggested that normal sera contain platelet-derived inhibitors of megakaryocyte colony formation.23,24 However, it is unlikely that such inhibitors, if present, substantially influenced our experimental results because both peak megakaryocyte colony formation and maximal nuclear polyplody were achieved at a concentration ratio of aplastic canine to normal AB serum of less than 0.10. It is also improbable that the aplastic canine serum contained substantial quantities of platelet-derived inhibitors, given the low platelet count at the time of its preparation. We have recently established that the megakaryocyte colony-stimulating activity manifested by aplastic canine sera results from the presence of a positive regulatory protein rather than from the absence of inhibitors found in normal sera.6

By examining the polyplodyization of human megakaryocyte progenitors in culture, our studies represent an extension of the previously published murine in vitro studies.11,12,15-17,22 Our use of immunochemical labeling of cultured human megakaryocyte colonies for identification with concurrent Feulgen-BAO staining in situ for DNA quantitation is analogous to those murine studies in which megakaryocyte colonies were double labeled in situ for acetylcholinesterase and DNA.15-17 Such approaches provide unequivocal identification of all megakaryocytes present within a culture (including low-ploidy cells) and eliminate both the biases inherent in morphological identification using inverted microscopy and the cell losses that accompany colony transfer to glass slides for DNA analysis. The use of Feulgen-BAO staining coupled with microfluorometry for the determination of the cellular DNA content represents a technically straightforward and accurate approach to DNA quantitation, one that does not require the expensive equipment necessary for scanning microdensitometry. Furthermore, distributional errors, excessive signal-to-noise ratio, and light scatter are minimized or eliminated.10,25 Imprecision resulting from photodecomposition of the DNA fluorochrome is minimized by using BAO for which the fluorescent signal is relatively stable to the UV incident light used for excitation.9

This investigation also examined the relationship between cultured megakaryocyte ploidy and diameter. The positive correlation we observed (Fig 5) is similar to that previously described for megakaryocytes harvested directly from bone marrow.14 Our data defined a smaller megakaryocyte diameter for each ploidy class than that described by these other investigators. Possible explanations for this discrepancy include cell shrinkage due to fixation or the failure of the cultured megakaryocytes to achieve full cytoplasmic maturation in vitro. Nevertheless, this positive correlation demonstrates that the link between ploidy and cytoplasmic development previously found to exist in vivo is maintained during in vitro megakaryocyte development.

The availability of megakaryocyte progenitor cell culture systems in which one can concurrently quantify colony formation, cell numbers per colony, and endoreduplication provides new opportunities for analyses of megakaryocytopoietic regulation. Our data indicating that the mitotic history of a megakaryocyte precursor may influence its endoreduplicative potential serve to emphasize both the complexity of this regulatory system and the potential utility of the methods described in this investigation.

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