Analysis of Megakaryocyte Ploidy in Rat Bone Marrow Cultures

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Megakaryocytes undergo changes in ploidy in vivo in response to varying demands for platelets. Attempts to study the putative factor(s) regulating these ploidy changes have been frustrated by the lack of an appropriate in vitro model of megakaryocyte endomitosis. This report describes a culture system in which rat bone marrow is depleted of identifiable megakaryocytes and enriched in their precursor cells. Morphologically identifiable megakaryocytes appear when the depleted marrow is cultured in vitro. The total number of nucleated cells, as well as the number of megakaryocytes and their ploidy distribution, are quantitated very precisely by flow cytometry. Although the total number of nucleated cells declines by 35% to 40% over 3 days in culture, the number of megakaryocytes rises 10-fold. The number of nucleated cells, the number of megakaryocytes, and the extent of megakaryocyte ploidy distribution behave as independent variables in culture and are dependent on the culture conditions. The addition of recombinant erythropoietin promotes a rise in the number of megakaryocytes and a shift in ploidy to higher values while recombinant murine granulocyte-macrophage colony stimulating factor is without effect on the cultured megakaryocytes. This in vitro system may provide a means to study those factors that affect megakaryocyte growth and ploidy.

THE MEGAKARYOCYTE is unique among bone marrow cells in possessing a polyploid nucleus that contains four (8N), eight (16N), 16 (32N), or even 32 (64N) times the diploid amount of DNA. At some stage following commitment to the megakaryocyte lineage, the megakaryocyte precursor cell ceases to undergo cytokinesis but continues to replicate its DNA. This process, called endomitosis, results in megakaryocyte precursors of high ploidy but with little identifiable cytoplasmic maturation. With the completion of endomitosis, the cells undergo massive cytoplasmic development and eventually produce platelets.

The regulation of megakaryocyte endomitosis has been studied almost exclusively in vivo using Feulgen staining or flow cytometry of bone marrow to identify megakaryocytes and quantify their DNA content. These studies have shown that both megakaryocyte ploidy and the number of megakaryocytes increase or decrease in response to a respective greater or lesser demand for platelets. The maximum changes in megakaryocyte ploidy precede by several days the changes in the number of megakaryocytes and have been presumed to be under the influence of humoral factors. Since ploidy is a unique characteristic of the megakaryocyte, changes in ploidy should provide an excellent marker for the identification and purification of the putative humoral factors that regulate megakaryocyte growth. Unfortunately, the limitations imposed by such in vivo studies prohibit a more detailed analysis of megakaryocyte endomitosis and frustrate attempts to use in vivo changes in ploidy as an assay for these regulatory factors.

To overcome these difficulties, several different approaches have been used to analyze megakaryocyte endomitosis in vitro. Most of these methods use colony-forming unit-megakaryocyte (CFU-MK) colonies that are stained for DNA quantitation by Feulgen or chromomycin A₃ or used as a source of megakaryocyte precursor cells for single cell studies. Other methods use liquid cultures of whole murine bone marrow which are subsequently Feulgen stained to quantitate megakaryocyte growth. Unfortunately all of these methods are limited by the cumbersome methods used to quantitate DNA. Moreover, methods dependent on CFU-MK are handicapped by the small number of cells available for study, while the liquid culture method is unable to distinguish megakaryocytes that appear during culture from those that have persisted from the initial marrow plating.

This report describes a new approach by which megakaryocyte endomitosis may be studied in vitro. With this method most morphologically identifiable megakaryocytes are removed from rat bone marrow and the residual marrow, enriched in megakaryocyte precursors, is placed in liquid culture. Over the next several days, megakaryocytes grow and their number and ploidy are measured very precisely by flow cytometry (FCM). This culture method may be useful in the identification of humoral substances regulating megakaryocyte growth and differentiation.

MATERIALS AND METHODS

Reagents and Animals

Trisodium citrate was purchased from Mallinckrodt (Paris, KY). Propidium iodide, adenosine (free base), theophylline, deoxyribonucleic acid (type IV), avdin, ribonucleic acid (type I-A), and hemoglobin standard were obtained from Sigma (St Louis, MO). Potassium cyanide was obtained from Aldrich (Milwaukee, WI); potassium ferricyanide was from Fisher (Medford, MA). Percoll and cyagen bromide-activated Sepharose 6MB were obtained from Pharmacia (Piscataway, NJ). Young (250 to 300 g) and retired breeder (600 to 800 g) Sprague-Dawley-derived (CD) rats were obtained from Charles River Breeding Laboratories, Inc (Wilmington, MA) and...

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were housed in single cages with free access to food and water for at least 1 week before use. Highly purified human recombinant erythropoietin was a generous gift of Genetics Institute (Cambridge, MA). Murine recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) was purchased from Genzyme Corp (Boston, MA).

Preparation of Rat Sera

Under ether anesthesia, rats were bled by cardiac puncture into citrate (final concentration, 0.38%). The citrated whole blood (WB) was centrifuged at 700 g for 8 minutes at 4°C to prepare platelet-rich plasma (PRP). PRP or citrated WB were centrifuged at 3,000 g for 15 minutes at 4°C to prepare platelet-poor plasma (PPP). Rat serum (RS), rat PRP-derived serum (RPRPDS), and rat PPP-derived serum (RPPPDS) were obtained from rat WB, PRP, and PPP, respectively, by recalcification in glass tubes and incubation at 37°C for 1 hour. Clots were removed and the sera heated at 56°C for one-half hour. All samples were stored at -80°C and retained full growth-promoting or growth-inhibiting activity for over 3 years.

Preparation of Megakaryocyte-Depleted Bone Marrow

Using avidin-sepharose affinity chromatography. With sterile technique, bone marrow was expressed from the femurs of a 600-g rat into 9 ml of CATCH buffer. Unless otherwise stated all CATCH solutions contained DNase (0.02 mg/ml, 37 Kunitz U/ml) and were prepared as described by Jackson et al.7 Marrow was disaggregated three times with an 18 gauge needle, then times with a 10 ml pipette and large particles allowed to settle for 30 seconds. The supernatant was then removed, centrifuged at 380 g for 5 minutes at 4°C, and the cells resuspended in 9 ml of CATCH. Following a second centrifugation, the cell pellet was resuspended in lysis buffer (0.14 mol/L NH4Cl, 0.017 mol/L Tris, pH 7.2) and incubated at 37°C for 10 minutes.7 (This step resulted in the lysis of all red blood cells (RBCs) and improved recoveries from the subsequent Percoll gradient.) Cells were washed twice by centrifugation and were resuspended in CATCH. The suspension was filtered through a 250 μm mesh, diluted to 15 ml with CATCH, and centrifuged for 20 minutes at 380 g at room temperature over a discontinuous Percoll gradient (15 ml of 1.060 g/cm3 Percoll over 15 ml of 1.085 g/cm3 Percoll made in CATCH without DNase, pH 7.40, 325 mOsm/kg). The cells at the 1.060:1.085 g/cm3 interface were collected, diluted to 45 ml with CATCH, and centrifuged at 380 g for 10 minutes. The cell pellet was resuspended in CATCH, centrifuged again, and resuspended in 9 ml of CATCH containing anti-platelet serum (APS). After one-half hour incubation at 4°C, the cells were washed once with CATCH and then incubated one-half hour at 4°C with biotin-conjugated goat anti-antibody IgG (Cappel, Malvern, PA). After washing twice with CATCH, the cells were resuspended to a volume of 5 ml. Using an avidin-Sepharose affinity column prepared exactly as by Berenson et al24 and equilibrated with 5 column volumes of CATCH, the cell suspension was loaded at a flow rate of 0.5 ml/minute and eluted with 3 column volumes of CATCH. The effluent cells were concentrated by centrifugation, washed once with CATCH, and the final pellet resuspended to a density of 20 x 10⁶ cells/ml in culture medium.

Using Percoll density gradient centrifugation and filtration. Bone marrow was prepared as in the previous section through the collection of the 1.060:1.085 interface cells. These cells were digested to 45 ml with CATCH and centrifuged at 380 g for 10 minutes. The cell pellet was resuspended in CATCH, centrifuged again, and resuspended to a density of approximately 25 x 10⁶ cells/ml in culture medium. The suspension was filtered twice through a sterile 17-μm polyester mesh (Tetko, Inc, Elmsford, NY) and was then ready for culture.

Using counterflow centrifugal elutriation. Marrow was prepared as by Greenberg et al.7 The cells washed out of the elutriator chamber at a buffer flow rate of 12 to 15 ml/min were collected and concentrated by centrifugation. To remove residual RBCs and megakaryocytes, the cell pellets were resuspended in a solution of Percoll diluted with Iscove's modification of Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) to a final density of 1.050 g/cm³. A discontinuous gradient of three layers was prepared: 10 ml of CATCH buffer, 15 ml of the cell suspension in 1.050 g/cm³ Percoll, and 15 ml of 1.085 g/cm³ Percoll in CATCH buffer. The gradients were centrifuged at 180°C for 20 minutes at 400 g and the cells at the 1.050:1.085 g/cm³ interface were collected, diluted fourfold, and washed once by centrifugation in CATCH to remove the Percoll. The final pellet was resuspended to a density of 20 x 10⁶ cells/ml in culture medium.

Bone Marrow Culture

Unless otherwise stated, bone marrow was grown in IMDM containing penicillin (200 U/mL), streptomycin (200 μg/mL), and additional glutamine (0.592 mg/mL) in the presence of 20% horse serum (GIBCO). Compared with RPMI and Dulbecco's modified essential medium (DMEM), IMDM provided the best culture medium. Cells were routinely cultured at a final density of 7 x 10⁶ cells/mL in a total volume of 3 ml in 6-well (40 mm) plates (Costar, Cambridge, MA) for 3 days at 37°C in a 5% CO₂ incubator. From one bone marrow preparation 10 to 15 wells for culture were usually obtained. Subsequently wells were harvested by removing the media and cells, rinsing with 1 ml CATCH, and centrifuging the pooled cell suspensions at 180 g. The cell pellet was resuspended to a volume of 3 ml in CATCH and stained for flow cytometric analysis as described below. Adherent cells remaining in the wells following harvesting were less than 1% of the total cells recovered.

Preparation of APS

APS was prepared as described by Jackson et al.7 Intravenous injection of APS into rats produced profound thrombocytopenia but no change in the hematocrit or white blood cell (WBC) count. Staining of rat bone marrow suspensions with APS (vide infra) produced intense staining of all morphologically identifiable megakaryocytes and only occasional, very faint staining of other bone marrow cells.

Harvesting Normal Rat Bone Marrow Cells for FCM

Rats were anesthetized with ether, bled approximately 50% to 60% of their blood volume by direct cardiac puncture, and the bone marrow expressed from both femurs into a final volume of 9 ml of CATCH. Marrow was disaggregated three times with an 18 gauge needle and large clumps allowed to settle for 30 seconds. The supernatant cells (approximately 30 x 10⁶ cells/mL) were used as controls in each FCM run.

Staining of Bone Marrow Cells

Bone marrow was stained by the method of Jackson et al7 but modified to minimize the number of centrifugations. APS or control normal rabbit serum (NRS) were added to the 3 ml cell suspension from cultured cells or from uncultured normal rat bone marrow, respectively, incubated for 30 minutes at 4°C, and the cells pelleted by centrifugation for 5 minutes at 380 g. Cells were suspended in 3 ml of fresh CATCH and recentrifuged. The pellet was then resuspended in 3 ml of CATCH containing 75 μL of fluorescein-conjugated goat
anti-rabbit IgG (Cappel, Malvern, PA) and incubated at 4°C for one-half hour. The cells were then pelleted once by centrifugation, resuspended in 3 mL of CATCH, and centrifuged one last time. The final cell pellet was resuspended in 3 to 10 mL of propidium iodide solution (0.05 µg/mL in 0.10% sodium citrate, pH 7.40, 7 mOsm/ kg), which produces uniform nuclear staining of all cells. Cells were stored up to 24 hours at 4°C with no change in flow cytometry pattern. Just before FCM, the cells were resuspended by inversion of the tube and treated with ribonuclease (0.05 mg/mL, final concentration) for one-half hour at room temperature. Cells were filtered through a 100 µm mesh, counted by hemacytometer, diluted with propidium iodide solution to a concentration of 1.5 x 10^6/mL, and then run on the flow cytometer.

Hemoglobin Quantitation

Hemoglobin was quantitated by the cyanmethemoglobin method. Cell pellets from whole bone marrow or cultured marrow (10 to 20 x 10^6 nucleated cells) were suspended in 0.75 mL of Drabkin's solution, incubated 10 minutes at room temperature, and then centrifuged at 1,000 g for 5 minutes. The supernatant was removed and filtered through a 0.22-µm filter (Acrodisc LC13; Gelman Scientific Inc, Ann Arbor, MI). The absorbance at 540 nm was then read and compared with hemoglobin standards.

Flow Cytometry

Flow cytometry was performed on a machine designed and built by H.M. Shapiro. A Laser Ionics (Trimedyn Laser Systems, Inc, Orlando, FL) 1 W argon ion laser was used usually at a power output of 250 mW. Light was focused through 250 mm focal length and 60 mm focal length cylindrical lenses before passing through the flow chamber capillary (100 to 170 µm inner diameter). Emitted fluorescence was collected with an 18 x (numerical aperture 0.45) lens and passed through a 560 nm dichroic filter. The orange fluorescence signal was then passed through O.G 570 and WB 600 filters covering the photomultiplier tube while the green fluorescence signal was filtered further through OG 515 (Schott Optical Glass, Duryea, PA) and 535 nm wide band (WB) pass interference (Melles Griot, Irving, CA) filters covering the photomultiplier tube. A coefficient of variation (CV) of the 2N peak was maintained at 2.2% to 3.0% by careful alignment of the optical system. To define the megakaryocyte population the "lower green fluorescence window" was established based on the observation under the fluorescence microscope that a subset of the uncultured bone marrow megakaryocytes stained faintly with NRS. Control uncultured bone marrow samples stained with NRS were routinely analyzed by the flow cytometer at the beginning of each run and the 16N ploidy group detected. The gain on the green photomultiplier tube was altered to keep the maximum fluorescence intensity of these 16N megakaryocytes at 10% of the full oscilloscope scale. The lower green fluorescence window was then set to exclude all cells with fluorescence intensity less than 10% of the fluorescence scale. Cells were routinely run at 800 to 1,200 cells per second and 2,000 megakaryocytes were analyzed. All ploidy classes were assigned at the start of each run from the DNA histogram of APS stained, uncultured bone marrow.

Data Analysis

Data from the flow cytometer were stored and analyzed on an Atari 130 XE computer (Atari Corp, Sunnyvale, CA). Due to a software limitation in the flow cytometer used for these studies, the 2N and 4N cells could not be adequately counted. Only megakaryocyte classes 8N and greater were analyzed and the total number of megakaryocytes ≥ 8N in the sample was expressed as a percentage of the total cells counted by the flow cytometer. Each ploidy group was represented as a percentage of the total number of megakaryocytes ≥ 8N. The geometric mean of each ploidy distribution was calculated as described by Arriaga et al. 18

Except for the ploidy distributions, statistical analysis of differences was performed using Student's t test. Differences between ploidy distributions were analyzed by the Mann-Whitney U Test.

RESULTS

Preparation of Megakaryocyte-Depleted Rat Bone Marrow

In preliminary experiments, whole rat bone marrow was placed into IMDM and cultured for 3 days at 37°C. When the cultured bone marrow was analyzed by FCM, the average ploidy of the megakaryocytes was lower than the starting marrow but the total number of megakaryocytes was unchanged or slightly increased. This therefore suggested that new megakaryocytes were entering the megakaryocyte population during culture. Since it was not possible to distinguish the new megakaryocytes from those persisting from the starting bone marrow, efforts were undertaken to deplete the starting bone marrow of all morphologically identifiable megakaryocytes so as to permit detection of those that appeared during culture.

Megakaryocytes were successfully removed from rat bone marrow by elutriation, avidin-sepharose affinity chromatography, or Percoll density gradient centrifugation and filtration. Starting with approximately 400 x 10^6 nucleated bone marrow cells from one rat, all three methods provided satisfactory material for culture and differed only in recovery, convenience, and the extent of clearance of the megakaryocytes. When the numbers of nucleated cells and morphologically identifiable megakaryocytes were monitored by hemacytometer, avidin-sepharose affinity chromatography (average of eight experiments) was found to remove 99.0% ± 0.7% of the megakaryocytes with recovery of 25.0% ± 8.0% of total nucleated cells but was the most time-consuming of the methods. Elutriation (average of 18 experiments) removed 92.4% ± 6.7% of the megakaryocytes with a recovery of 27.0% ± 8.0% of total nucleated cells but required almost as much time. The Percoll density gradient centrifugation and filtration method (average of 48 experiments) was much easier and reproducibly removed 94.0% ± 4.3% of the megakaryocytes with a recovery of 41.0% ± 11.0% of all nucleated cells. The results described in subsequent experiments were the same regardless of the depletion method used.

The effect of megakaryocyte depletion on rat bone marrow is illustrated in Figs 1 and 2. Starting bone marrow contained numerous morphologically identifiable megakaryocytes (Fig 1A) that could no longer be found following depletion (Fig 1B). This depletion could be precisely quantitated by FCM: the starting bone marrow (Fig 2A) contained 0.19% megakaryocytes ≥ 8N with a ploidy distribution of 24% 8N, 60% 16N, and 16% 32N, whereas following depletion (Fig 2B) there were 0.006% megakaryocytes ≥ 8N with a ploidy distribution of 41% 8N, 39% 16N, and 20% 32N. Over 96% of the megakaryocytes identified by FCM were removed with a recovery of total nucleated cells of 25%.

Culture of Megakaryocyte-Depleted Bone Marrow

When the megakaryocyte-depleted bone marrow cells were cultured in IMDM in the presence of horse serum
Fig 1. Phase contrast microscopy (100 X) of rat bone marrow cultures (7 x 10^6 cells/mL). (A) Normal rat bone marrow before depletion. Arrows indicate megakaryocytes. (B) Bone marrow after depletion by avidin-sepharose affinity chromatography. (C) Depleted bone marrow after 3 days of culture.
Fig 2. Two-dimensional FCM fluorescence plot of rat bone marrow. The abscissae indicate DNA content (orange fluorescence) on a linear scale and the ordinates indicate APS binding (green fluorescence) on a linear scale. The total number of cells analyzed (5 \times 10^6) for each preparation is the same. (A) Normal rat bone marrow before depletion. (B) Bone marrow after depletion by avidin-sepharose affinity chromatography. (C) Depleted bone marrow after 3 days of culture.

(HS), the total number of nucleated cells was unchanged for 24 hours, but thereafter declined steadily for 5 days (Fig 3). However, over that same period morphologically identifiable megakaryocytes appeared in the culture and increased in number (Fig 1C). After staining with anti-platelet serum

and propidium iodide, the total number of nucleated cells and the percentage of megakaryocytes and their ploidy distribution could be quantitated very accurately by FCM. Figure 2C shows the FCM pattern of the cells pictured in Figure 1C: all ploidy groups were present and easily quantitated. There were 0.215% megakaryocytes \( \geq 8N \) with 25% 8N, 57% 16N, and 18% 32N. (Although the percentage of megakaryocytes and the ploidy distribution before depletion are virtually identical to those after culture, this is coincidental. As noted below, the ultimate number and ploidy distribution of megakaryocytes after culture are solely dependent on the culture conditions, not on the composition of the starting bone marrow.) Except for diminished average immunofluorescence intensity at each ploidy class, the cultured megakaryocytes appeared the same by FCM as those megakaryocytes taken directly from the animal.

Over 6 days in culture the changes in the total number of megakaryocytes and in the ploidy distribution are shown in Fig 4. The total number of megakaryocytes \( \geq 8N \) rose linearly from day 0 to day 3 and this rise (data not shown) continued until day 5. By day 6, with no change of medium, the cells started to die. When individual ploidy groups were analyzed, the 8N and 16N ploidy groups were found to increase over the first 24 hours while the 32N group rose only after a 24-hour lag. From day 1 until day 3 the 8N rose somewhat slower and the 16N a bit more rapidly. For each of the three ploidy groups the number of cells rose linearly until day 5 (data not shown) and then declined.

On average, one bone marrow preparation from two rats provided enough marrow for 10 to 15 separate cultures. A standard duration of 3 days was arbitrarily chosen for routine bone marrow cultures. Over this period of time, cultures (average of three experiments) seeded with 15.5 ±
average ploidy as the serum concentration rose. On the basis of these results, 20% HS was chosen for routine use.

**Effect of Serum Versus Plasma on Cultures**

To determine why RS was such a poor stimulant for megakaryocyte growth, the effect of RS on bone marrow cultures was compared with that of RPPPDS and HS (Table 1). There was no statistically significant difference in the recovery of nucleated cells between cultures grown in these serum sources. However, RS produced on the average only 39% as many megakaryocytes with a relative mean ploidy 45% of that of cultures grown in HS. RPPPDS was intermediate in its effect: it stimulated 67% as many megakaryocytes with a mean relative ploidy 60% as much as that of cultures grown in HS. To determine whether the different effects of RS and RPPPDS were mediated by the presence of inhibitors, a series of 1:1 mixes of between HS and RS or RPPPDS were performed and the effect on cultures tested. Compared with HS, both mixes demonstrated slightly increased total cell number (not statistically significant but probably reflecting the increased total serum concentration in the 1:1 mixes) and both showed the same identical decrease in total megakaryocytes as seen in cultures grown with the unmixed RS or RPPPDS. But while the HS/RS mix showed decreased relative mean ploidy, the HS/RPPPDS mix showed a statistically significant increase in ploidy (but identical to that found with 30% HS).

**Effect of Serum Concentration on Cultures**

Megakaryocyte-depleted cultures were found to grow maximally in the presence of HS. Rat serum and fetal calf serum gave much poorer stimulation of megakaryocyte growth. To determine the optimal HS concentration, cultures were grown for 3 days in various concentrations of HS and the cultures analyzed for total nucleated cell number, total number of megakaryocytes, and megakaryocyte ploidy. The total number of nucleated cells remaining in culture increased linearly (from $12 \times 10^6$ to $19 \times 10^6$ cells per well) as the serum concentration rose from 5% to 30%. However, the total number of megakaryocytes $\geq 8N$ in culture (Fig 5) reached a plateau at 15% HS while the megakaryocyte ploidy distribution in culture showed a continuous increase in...
culture became visibly hemoglobinized and the erythropoiesis surviving 3 days in culture (Fig 6A). Moreover, the cells in concentration-dependent increase in total nucleated cells and 32N megakaryocytes rose by twofold and eightfold, respectively.

When individual ploidy classes were analyzed (Fig 7) at an erythropoietin concentration of 5 U/mL the proportion of 8N megakaryocytes declined by two-thirds while the 16N and 32N megakaryocytes rose by twofold and eightfold, respectively.

The addition of recombinant murine GM-CSF to the cultures produced an increase in the total number of nucleated cells, the rise in total nucleated cells (data not shown). When analyzed by FCM, erythropoietin produced a more than twofold increase in total megakaryocytes ≥8N (Fig 6B).

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The addition of recombinant murine GM-CSF to the cultures produced an increase in the total number of nucleated cells beginning at approximately 10 U/mL and rising continuously to 100 U/mL (Fig 6A). In the absence of erythropoietin the cultures never became hemoglobinized. Moreover, when analyzed by FCM there was no increase in the total number of megakaryocytes (Fig 6B) nor any change in ploidy distribution (data not shown) over this range of GM-CSF concentrations.

**Effect of Recombinant Erythropoietin and GM-CSF on Cultures**

On addition of erythropoietin to cultures there was a concentration-dependent increase in total nucleated cells surviving 3 days in culture (Fig 6A). Moreover, the cells in culture became visibly hemoglobinized and the erythropoiesis concentration-dependent rise in hemoglobin paralleled the rise in total nucleated cells (data not shown). When analyzed by FCM, erythropoietin produced a more than twofold increase in total megakaryocytes ≥8N (Fig 6B).

When individual ploidy classes were analyzed (Fig 7) at an erythropoietin concentration of 5 U/mL the proportion of 8N megakaryocytes declined by two-thirds while the 16N and 32N megakaryocytes rose by twofold and eightfold, respectively.

The addition of recombinant murine GM-CSF to the cultures produced an increase in the total number of nucleated cells beginning at approximately 10 U/mL and rising continuously to 100 U/mL (Fig 6A). In the absence of erythropoietin the cultures never became hemoglobinized. Moreover, when analyzed by FCM there was no increase in the total number of megakaryocytes (Fig 6B) nor any change in ploidy distribution (data not shown) over this range of GM-CSF concentrations.

**Accuracy and Reproducibility of Cultures**

From all cultures three parameters have been routinely analyzed by FCM: the total number of nucleated cells, the number of megakaryocytes ≥8N, and the megakaryocyte ploidy distribution. A review of the first 200 bone marrow preparations (approximately 2,000 separate cultures) permitted an analysis of the accuracy and reproducibility of this method. Within any one preparation, replicate cultures grown in the same serum source showed very little well-to-well variation: the CV for the total number of nucleated cells was 8% while the CV for the number of megakaryocytes was 3.5% and that for the percentage of cells in each ploidy class was 2%. Between different bone marrow preparations, however, cultures grown with the same serum source showed somewhat greater variation: the CV for the total number of nucleated cells was 8% while the CV for the number of megakaryocytes and the percentage in each ploidy class was each 10%.

**DISCUSSION**

To study the regulation of megakaryocyte ploidy, a reliable in vitro system is needed to permit detailed analysis of the endomitotic process. Since none of the available methods proved well-suited for this purpose, an alternative bone marrow culture method was devised. This new method is based on the finding that bone marrow contains both early and late megakaryocyte precursor cells. While the latter cells are usually morphologically identifiable, the former, consisting of cells such as the CFU-MK and cells equivalent to the small acetylcholinesterase-positive cells of the rat, are usually not. Given the reported observation the megakaryocyte ploidy changes usually start to occur 24 hours after the development of thrombocytopenia and that the maturation time from diploid precursors to mature cells in rats is about 2 days, it would appear that the cell population that responds to thrombocytopenia is primarily the early precursor population. If this early precursor cell population could be isolated and cultured, it would be an ideal population with which to study endomitosis. Unfortunately, attempts to purify this population to homogeneity have not met with success. Therefore, a novel method has been developed in which most morphologically identifiable megakaryocytes have been removed from the bone marrow leaving behind, presumably, the morphologically indistinct precursors mixed with a majority of nonmegakaryocyte bone marrow cells. On culture, morphologically identifiable megakaryocytes appear and their number and ploidy distribution may easily be quantitated with great precision by FCM. Similar methods of bone marrow depletion have been used to analyze WBC and RBC maturation in culture.

While the Percoll density gradient centrifugation and filtration method is recommended because of its conve-
nience, several techniques to deplete megakaryocytes from bone marrow have been compared. All produced megakaryocyte-depleted bone marrows that grew equally well in culture. All used a Percoll gradient that helped to isolate a cell population with density between 1.060 and 1.085 g/cm³, a density at which few mature megakaryocytes are found and at which most CFU-MK and small acetylcholinesterase-positive cells are retrieved. While the avidin-sepharose affinity chromatography method cleared away 99% of megakaryocytes, such extensive removal of megakaryocytes was found not to be routinely necessary since marrow depleted by the Percoll method (which clears away only 94% of megakaryocytes) produced identical results on culture. The removal of the majority of megakaryocytes seems necessary, at the very least, to produce a sufficiently low number of background mature megakaryocytes to enable the newly grown megakaryocytes to be detected by FCM. In addition, the removal of these mature megakaryocytes may reduce a potential inhibitor of megakaryocyte growth as suggested by Gewirtz and Sacchetti.

Though preventable by the addition of erythropoietin and GM-CSF, routine cultures grown in the absence of added recombinant growth factors showed a decline with time in the total number of nucleated cells, presumably reflecting...
the death of myeloid and erythroid cells. However, the total number of megakaryocytes increased approximately 10-fold when quantitated by the FCM. Using a short-term liquid culture of whole murine bone marrow driven by pokeweed mitogen-stimulated spleen cell-conditioned medium (PWMSCM), Petursson and Chervenick have shown a similar decline in the total nucleated cells and rise in acetylcholinesterase-positive megakaryocytes.

With a similar system Nagasawa et al. found a fourfold increase in megakaryocytes during culture. As suggested by the results of Vainchenker et al., the much higher cell concentration used here with the megakaryocyte-depleted bone marrow presumably obviates the need for exogenous stimulators of megakaryocyte growth such as PWMSCM or purified interleukin-3. The megakaryocytes grown from culture appear morphologically identical to normal megakaryocytes and differ only in having less surface APS staining as measured by decreased immunofluorescence on the flow cytometer. It is unclear whether this simply reflects the relatively short length of time available for maturation in culture versus that in vivo or indicates the absence in vitro of factors required for cytoplasmic maturation as suggested by others.

After culture the bone marrow cells may be analyzed by FCM and three parameters quantitated: the total cell number, the number of megakaryocytes ≥ 8N, and the megakaryocyte ploidy distribution. FCM has been shown to be a highly accurate method of making these determinations in whole bone marrow and shows similar precision in analyzing the megakaryocytes in cultured bone marrow, as reported here.

To maximize the growth of megakaryocytes in culture, the effects of various sera were analyzed. HS was found to be the best stimulus of both megakaryocyte number and ploidy while RS was the poorest and RPPPDS was intermediate in effect. In exploring further the difference between RS and RPPPDS, both were found to contain inhibitory substances. RPPPDS inhibited the number of megakaryocytes that appeared in culture but had either a slightly stimulatory or no effect on megakaryocyte ploidy. On the other hand, RS inhibited the number of megakaryocytes to a much greater degree than RPPPDS and also inhibited megakaryocyte ploidy development in culture. Although it has long been noted by others that serum but not plasma inhibits CFU-MK growth, this is the first demonstration that serum also inhibits ploidization. The results of these mixing experiments also demonstrate that in this culture system the total cell number, the number of megakaryocytes ≥ 8N, and the ploidy distribution all behave as independent variables.

To assess the impact of growth factors in this system, the depleted bone marrow cultures were grown in the presence of recombinant human erythropoietin and recombinant murine GM-CSF. On the addition of erythropoietin, erythroid precursor cells were maintained in culture and became hemoglobinized. As the erythropoietin concentration rose, the total number of nucleated cells in culture and the total number of megakaryocytes increased while megakaryocyte ploidy was shifted toward higher values. While prior studies have shown that erythropoietin stimulates the number of CFU-MK as well as the size of megakaryocytes in single cell culture and in vivo, only the studies presented here have measured directly the effect of erythropoietin on the number and ploidy of megakaryocytes.

In contrast to the effect of recombinant erythropoietin on megakaryocytes, recombinant murine GM-CSF showed no effect on the number or ploidy distribution of megakaryo-
cytes despite causing a marked increase in the total number of nucleated cells in culture at concentrations from 10 to 100 U/mL. In most other studies, GM-CSF has been found to increase the number of CFU-MK,\(^5\) to synergize with IL-3,\(^6\) and in vivo to increase two- to fourfold the number of splenic megakaryocytes.\(^6\) Since the depleted bone marrow culture system mostly reflects effects on cells after the CFU-MK stage, the differences between these published results and those reported here are not surprising. In the only study where the effect of GM-CSF on megakaryocyte ploidy was assessed, Mazur et al\(^9\) also found no effect on the ploidy of megakaryocytes in CFU-MK.

By allowing the precise and reproducible quantitation of megakaryocyte number and ploidy in vitro this bone marrow culture system may provide a sensitive assay for the identification and purification of circulating factor(s) regulating megakaryocyte growth and differentiation. Moreover, the culture system described here should be useful in assessing the effect of recombinant growth factors such as erythropoietin or GM-CSF on megakaryocyte number and ploidy. By providing a *tabula rasa* at the start of culture, the effects on megakaryocyte-specific biochemical markers, such as acetylcholinesterase and PF4 mRNA, may also be quantitated during culture. Finally, in the absence of megakaryocyte-specific markers, cultured megakaryocytes may be removed after culture using botrocetin,\(^3,4,6\) and biochemical measurements performed (D.J. Kuter, unpublished observation). The wide range of applications for this culture system may therefore permit more direct analysis of megakaryocyte growth and differentiation in vitro than has been possible to date.

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Analysis of megakaryocyte ploidy in rat bone marrow cultures

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