Investigations linking human megakaryocyte development and cell biology have been hindered by an inability to obtain large, relatively pure megakaryocyte cell preparations from in vitro stem cell cultures. We report here that such preparations can be generated from liquid cultures of normal human peripheral blood mononuclear cells stimulated by a serum source of megakaryocyte colony stimulating activity (Meg-CSA, the 0% to 60% ammonium sulfate protein fraction of aplastic canine serum). Adherent-depleted peripheral blood mononuclear cells are suspended at 5 x 10^6 to 10^8 cells/mL in supplemented liquid culture medium, platelet-poor human plasma 20% (vol/vol) and 1 to 2 mg/mL serum Meg-CSA protein. After 12 to 14 days of incubation, megakaryocytes constitute 3.0 ± 2.9% (mean ± SD, n = 8) of the unseparated cultured cell population. Megakaryocytes can be enriched by counterflow centrifugal elutriation to a purity of 88 ± 14% ± SD) with a recovery of 13 ± 7% and a viability of 87 ± 19%. This algorithm results in the average isolation of approximately 3 x 10^6 enriched megakaryocytes from a 100-mL starting volume of peripheral blood. Cultured megakaryocytes exhibit normal light and ultrastructural morphology by Wright-Giemsa staining and electron microscopic analysis. After a 12-day culture interval, enriched megakaryocyte preparations exhibit morphologic stage distributions that are similar to normal human marrow. Stage distributions move rightward with culture duration indicating partial synchony of megakaryocyte maturation. On cytospin preparations, megakaryocyte diameter averages 30.2 ± 1.5 µm and increases with maturation stage. Flow cytometric analyses demonstrate the expression of platelet glycoproteins (GP) IIb and IIb/IIIa by the cultured megakaryocytes. The modal ploidy of the enriched cells at day 12 of culture is 16N and most remaining megakaryocytes are 8N or 32N. Liquid culture of serum Meg-CSA-stimulated human peripheral blood mononuclear cells represents a valuable investigative tool that should permit studies of human megakaryocyte biology that have not been possible in the past.

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megakaryocytopoiesis and in preliminary analyses of the determinants of megakaryocyte polyploidization.\textsuperscript{15,16} However, megakaryocytes grown in semisolid cultures are either permanently immobilized within the culture matrix or can be recovered only in limited quantities using the labor intensive process of visual colony identification and manual megakaryocyte extraction and pooling. Using this latter approach, Jenkins et al isolated up to \(10^6\) stem cell derived megakaryocytes (70% to 90% pure) from methylcellulose cultures; cells that were 95% viable and were shown to synthesize platelet glycoproteins IIb and IIIa.\textsuperscript{17}

In this investigation, we have developed a liquid culture system for human CFU-Meg and combined this with megakaryocyte separation algorithms to generate large, highly enriched populations of human megakaryocytes. Megakaryocytes are grown from normal human peripheral blood mononuclear cells in liquid cultures containing a serum source of Meg-CSA. This approach has permitted the routine isolation of greater than \(10^5\) stem cell derived megakaryocytes with purities of greater than 50% using an initial 100 mL sample of normal peripheral blood. The cultured megakaryocytes are morphologically normal, express platelet specific proteins, and exhibit the expected heterogeneity in DNA content. With the large number and purity of megakaryocytes available as well as the capacity to control the in vitro developmental environment, this technique should be a valuable tool for the study of human megakaryocytopoiesis and megakaryocyte cell biology.

**MATERIALS AND METHODS**

**Human Subjects**

Peripheral blood for megakaryocyte progenitor cell (CFU-Meg) cultures and normal AB plasma/serum were obtained by routine venipuncture from healthy adult donors, all of whom provided written informed consent.

**Serum Meg-CSA**

An established source of Meg-CSA was enriched from aplastic canine serum. The serum protein fraction containing Meg-CSA was prepared by ammonium sulfate salt precipitation at 60% saturation and desalting by gel chromatography as described.\textsuperscript{11}

**Liquid Suspension Cultures of Human CFU-Meg**

Normal human peripheral blood mononuclear cells were isolated by Ficoll 400-sodium diatrizoate (Pharmacia, Piscataway, NJ) density centrifugation. The mononuclear cell suspension was layered over an equal volume of fetal calf serum (FCS) and pelleted by slow centrifugation in order to remove platelets. Cells were resuspended in alpha medium containing 20% AB serum and plastic adherent polystyrene tissue culture dishes (Corning Glass Works, Corning, NY).

### Table 1. Purity and Yield of Megakaryocytes Grown in Liquid Suspension and Separated by Countercflow Centrifugal Elutriation

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Peripheral Blood Volume (mL)</th>
<th>No. of Cells</th>
<th>Total Cell No.</th>
<th>Total Mk No.</th>
<th>% Mk*</th>
<th>Mk Recovery (%)</th>
<th>Mk Viability (%)</th>
<th>Total Cell Recovery Post-Elutriation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>1.5 x 10^7</td>
<td>1.6 x 10^7</td>
<td>2.2 x 10^6</td>
<td>1.4</td>
<td>5.2 x 10^4</td>
<td>2.9 x 10^4</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>140</td>
<td>9.5 x 10^7</td>
<td>2.0 x 10^8</td>
<td>3.6 x 10^6</td>
<td>1.8</td>
<td>1.1 x 10^4</td>
<td>6.2 x 10^4</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>1.2 x 10^8</td>
<td>2.7 x 10^7</td>
<td>6.2 x 10^6</td>
<td>2.3</td>
<td>1.5 x 10^4</td>
<td>1.1 x 10^4</td>
<td>73</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>1.5 x 10^7</td>
<td>3.1 x 10^7</td>
<td>1.4 x 10^6</td>
<td>0.4</td>
<td>9.8 x 10^5</td>
<td>3.6 x 10^5</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>1.8 x 10^7</td>
<td>1.4 x 10^7</td>
<td>8.2 x 10^6</td>
<td>5.7</td>
<td>8.1 x 10^4</td>
<td>4.1 x 10^4</td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>4.5 x 10^7</td>
<td>3.5 x 10^7</td>
<td>5.7 x 10^6</td>
<td>1.6</td>
<td>7.8 x 10^4</td>
<td>3.9 x 10^4</td>
<td>50</td>
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<tr>
<td>7</td>
<td>20</td>
<td>3.0 x 10^7</td>
<td>3.2 x 10^7</td>
<td>5.7 x 10^6</td>
<td>1.8</td>
<td>7.8 x 10^4</td>
<td>6.1 x 10^4</td>
<td>79</td>
</tr>
<tr>
<td>8</td>
<td>85</td>
<td>7.5 x 10^7</td>
<td>4.5 x 10^7</td>
<td>4.1 x 10^6</td>
<td>9.2</td>
<td>3.6 x 10^4</td>
<td>2.5 x 10^4</td>
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<tr>
<td>9</td>
<td>45</td>
<td>4.5 x 10^7</td>
<td>2.3 x 10^7</td>
<td>NA</td>
<td>NA</td>
<td>1.3 x 10^5</td>
<td>6.0 x 10^4</td>
<td>46</td>
</tr>
</tbody>
</table>

Overall mean ± SD: 3.0 ± 2.9

Abbreviations: Mk, megakaryocyte; NA, not available.

*Percentage of megakaryocytes determined on Wright-Giemsa stained cyt centrifuge preparations.

### Table 2. Purity and Yield of Megakaryocytes Grown in Liquid Suspension and Separated by Immunomagnetic Bead Adherence

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Total Cell No.</th>
<th>Total Mk No.</th>
<th>% Mk*</th>
<th>Cell No.</th>
<th>No. Mk</th>
<th>% Mk*</th>
<th>Mk Recovery (%)</th>
<th>Mk Viability (%)</th>
<th>Total Cell Recovery Post-Separation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.7 x 10^8</td>
<td>NA</td>
<td>NA</td>
<td>5.3 x 10^6</td>
<td>8.1 x 10^3</td>
<td>15</td>
<td>NA</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td>11</td>
<td>3.8 x 10^6</td>
<td>1.7 x 10^8</td>
<td>4.5</td>
<td>4.5 x 10^6</td>
<td>2.7 x 10^6</td>
<td>67</td>
<td>&gt;100</td>
<td>67</td>
<td>53</td>
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<tr>
<td>12</td>
<td>10.5 x 10^6</td>
<td>1.5 x 10^8</td>
<td>1.4</td>
<td>2.4 x 10^6</td>
<td>3.1 x 10^6</td>
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<td>20</td>
<td>&gt;100</td>
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<tr>
<td>13</td>
<td>4.6 x 10^6</td>
<td>1.7 x 10^8</td>
<td>3.7</td>
<td>1.6 x 10^6</td>
<td>8.6 x 10^4</td>
<td>55</td>
<td>51</td>
<td>54</td>
<td>63</td>
</tr>
</tbody>
</table>

Overall mean ± SD: 3.2 ± 1.6

Abbreviations: Mk, megakaryocyte; NA, not available.

*Percentage of megakaryocytes determined on Wright-Giemsa stained cytocentrifuge preparations.

†When a data point exceeds 100%, a value of 100 is used in calculations of the means ± SD.
The adherent depleted mononuclear cell population was suspended at $5 \times 10^3$ to $10^4$ cells/mL in 10 to 30 mL volumes in tissue culture flasks (Corning Glass Works). No megakaryocytes were identified by light microscopy in the initial cell inoculum. The suspension medium consisted of 20% heparinized platelet poor human AB plasma in supplemented Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) with serum Meg-CSA protein at 1 to 2 mg/mL as the growth stimulator. The supplemented IMDM was prepared by adding each of the following nutrients to 100 mL of IMDM: 1.0 mL each of 100× MEM nonessential amino acids, 100× L-glutamine, and 100× minimal essential medium vitamins, 10% (wt/vol) deionized bovine serum albumin, and $10^{-3}$ mol/L alpha-thioglycerol (100× supplements obtained from GIBCO). Suspension cultures were incubated for 12 to 13 days (unless otherwise indicated) at 37°C in a 100% humidified atmosphere of 5% CO$_2$ in air.

Enrichment of Megakaryocytes From Liquid Cultures

Counterflow centrifugal elutriation. Cells from suspension culture were resuspended in a medium specifically designed to preserve megakaryocyte integrity (Mega-buffer). Mega-buffer is a modification of CATCH medium and consists of Ca$^{2+}$ and Mg$^{2+}$ free phosphate buffered saline (PBS) supplemented with 1 mmol/L adenosine, 2 mmol/L theophylline, 0.3% (wt/vol) bovine serum albumin (BSA), 1 mmol/L Na$_2$EDTA, and 0.8% ACD-A (NIH formula); pH 6.5, final osmolarity, 305 ± 5 osm/L.

CCE was performed using a Beckman J2-21 centrifuge equipped with a JE-6B elutriation rotor and a Sanderson separation chamber. Cultured cells were injected into the inlet line (using Mega-buffer as the eluent) at room temperature with a flow rate of 31 mL/min and a rotor speed of 2500 rpm. The pump speed was increased to 45 mL/min by 2 to 3 mL/min increments and 100 mL of effluent was collected at each flow rate. The final fraction ("chamber fraction") was collected.

Fig 1. Representative megakaryocytes obtained from liquid culture of normal human peripheral blood mononuclear cells. Cells were cultured for 12 days using canine serum Meg-CSA protein as the growth stimulator. Megakaryocytes were enriched by CCE, sedimented onto glass slides by cytocentrifugation, and stained with Wright-Giemsa. (A-D) Megakaryocytes of differing morphologic stage: A, late stage I; B, stage II (right) and stage III (left); C, late stage III; D, stage IV. (Original magnification × 313.)
consisted of the noneluted cells and was collected by aspirating the contents of the Sanderson elutriation chamber. Megakaryocytes were retained in the chamber fraction although rare morphologically identifiable megakaryocytes (<1% of total) eluted in the final two fractions (corresponding to flow rates of 42 and 44 mL/min).

**Immunomagnetic bead adherence.** Cultured cells were washed once and resuspended in Mega-buffer with 0.1% sodium azide. Anti-platelet glycoprotein (GP) IIb/IIIa (AMAC, Westbrook, ME) was added at a 1:100 dilution and incubated with frequent agitation at 4°C for 30 minutes. After washing the cells twice, spherical immunomagnetic beads (Dynabeads; Dynal, Great Neck, NY) were added at a bead to cell ratio of 1:10. This resulted in approximately 3 to 10 beads per anticipated target megakaryocyte. The bead/cell mixture was incubated with frequent agitation at 4°C for 60 minutes. Cells bound to the immunomagnetic beads were collected by a magnetic particle concentrator (Advanced Magnetics, Cambridge, MA).6-10

**Megakaryocyte Analyses.**

Megakaryocyte Analyses

For determinations of megakaryocyte frequency, stage, and size, cells from the elutriation chamber fraction were mounted onto poly-L-lysine coated glass slides by cytocentrifugation (Shandon Cytospin 2, Sewickley, PA). Slides were Wright-Giemsa stained and megakaryocytes were identified by their characteristic light microscopic morphology. Megakaryocyte frequencies were determined by evaluating 363 to 3,945 (mean, 2,056) consecutive cells on the eyepiece micrometer. Megakaryocytes were assigned to maturation stages I through IV using the criteria of Levine et al.20,21

For electron microscopy, elutriation enriched megakaryocytes were suspended in Karnovsky’s fixative (5% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L cacodylate buffer, pH 7.2) for 30 minutes at 4°C, then pelleted by centrifugation and fixed overnight at 4°C. Fixed cells were washed in cacodylate buffer, post-fixed in osmium tetroxide, embedded in Epon, and stained with uranyl acetate and lead citrate at the Brown University core electron microscopy facility.

Megakaryocyte surface membrane antigen expression and DNA content were evaluated using flow cytometry. Megakaryocytes were enriched by CCE and stained immunofluorescently in Mega-buffer containing 0.1% sodium azide at 4°C. Cells were double labeled with a primary murine monoclonal antibody and fluorescein tagged goat F(ab)’, anti-mouse IgG (TAGO, Burlingame, CA). Monoclonal antibodies directed against platelet GP Ib (AP-1)22 and the IIb/IIIa complex (AP-2)23 were generously provided by Dr. R. Montgomery of The Blood Center of Southeastern Wisconsin. In addition, an anti-platelet GP IIb/IIIa murine monoclonal antibody (clone P2) was purchased from AMAC, Inc. Following immunofluorescent

### Table 3. Size and Morphologic Stage Distribution of Megakaryocytes Grown in Liquid Culture for 12 to 13 Days and Enriched by CCE

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of Mk* Evaluated</th>
<th>Morphologic Stage†</th>
<th>Mean Diameter‡ (µmol/L ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>I 2</td>
<td>2.3 ± 4.8</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>II 8</td>
<td>3.2 ± 7.6</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>III 7</td>
<td>3.3 ± 4.7</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>IV 2</td>
<td>1.5 ± 3.3</td>
</tr>
<tr>
<td>5</td>
<td>302/102</td>
<td>23</td>
<td>2.3 ± 4.8</td>
</tr>
<tr>
<td>6</td>
<td>206/152</td>
<td>7</td>
<td>2.3 ± 4.8</td>
</tr>
<tr>
<td>7</td>
<td>206/159</td>
<td>10</td>
<td>2.3 ± 4.8</td>
</tr>
</tbody>
</table>

Overall mean ± SD

<table>
<thead>
<tr>
<th>Mean Diameter‡ (µmol/L ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.4 ± 7.1</td>
</tr>
<tr>
<td>35.1 ± 4.8</td>
</tr>
<tr>
<td>49.9 ± 8.8</td>
</tr>
<tr>
<td>6.6 ± 3.3</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not determined; Mk, megakaryocytes.
*Where two numbers appear, they represent respectively the numbers of megakaryocytes evaluated for morphologic stage and the total number analyzed for size.
†Values represent the percentage of total megakaryocytes in each maturation stage. Numbers in parentheses are the mean diameters (±SD) for megakaryocytes segregated by maturation stage.
‡Mean diameter (±SD) of all megakaryocytes independent of stage.

Fig 2. Size distribution histogram of liquid culture derived megakaryocytes. After a 12- to 13-day culture interval, megakaryocytes were enriched by counterflow centrifugal elutriation, mounted on glass slides by cytocentrifugation, and stained with Wright-Giemsa. Diameters were calculated as the average of perpendicular measurements and cells were assigned to size classes at 5 µm intervals. Data are derived from three separate experiments incorporating measurements on a total of 413 megakaryocytes.
staining, cells were washed through FCS, incubated at 4°C for 45 minutes in Mega-buffer with 0.05% Tritox X-100 (Sigma, St Louis, MO), and fixed using 0.5% paraformaldehyde in Mega-buffer at 4°C for 5 minutes. Fixed cells were resuspended in propidium iodide at 25 μg/mL, 0.1% sodium citrate, and 1% BSA in saline and then stored for up to several days at 4°C. Just before flow cytometric analyses, samples were incubated with RNAase at 250 μM for 5 minutes. Fixed cells were resuspended in propidium iodide at 25 pg/mL for 30 minutes at room temperature. Cells were analyzed on an Epics Profile I (Coulter Cytometry, Hialeah, FL) equipped with a 15 mW Cyonics argon laser (Uniphase, San Jose, CA) and a 250 μm flow nozzle and cell. Data were acquired at a flow rate of 12 μL/min using log amplification, the green photomultiplier set at 690 V, and the red photomultiplier set at 580 V.

A positive green staining threshold was established using the megakaryocyte-free cultured lymphocytes which separated in the first elutriation fraction. Lymphocytes were stained identically to and concurrently with the megakaryocyte enriched fraction except that an irrelevant murine monoclonal antibody was substituted for the anti-platelet GP IIb/IIIa. The green gate was set such that the control lymphocyte population generated 0.6% to 0.7% positive events. This algorithm resulted in virtually 100% inclusion of all megakaryocytes ≥8N.

RESULTS

Normal appearing megakaryocytes routinely developed in serum Meg-CSA stimulated liquid suspension cultures established with adherent depleted normal human peripheral blood mononuclear cells. After 12 to 13 days of incubation, the megakaryocytes in such cultures comprised an average of 3% of the total cell population (Tables 1 and 2). In control day 12 cultures established without serum Meg-CSA, the megakaryocyte frequency was only 0.3 ± 0.1% (±SD, n = 4). Culture derived megakaryocytes appeared morphologically normal on Wright-Giemsa stained cytocentriuge preparations and exhibited varying size, stages of maturation, and extents of nuclear lobation (Fig 1).

In order to facilitate most analyses, megakaryocytes were enriched from the liquid cultures using counterflow centrifugal elutriation. Enrichment averaged 36-fold resulting in final megakaryocyte frequencies of 36% to 73% and a mean viability of 67% (Table 1). Total cell recovery following CCE averaged 61 ± 18% (±SD) while the mean megakaryocyte recovery was only 12.8 ± 6.9% (±SD). Normalized by the volume of peripheral blood used for the culture inoculum, this algorithm resulted in the isolation of 2.9 ± 10^5 (±1.9 ± 10^5, [±SD]) megakaryocytes with an average purity of 58% from each normal blood donation of 100 mL.

In an attempt to improve on the low megakaryocyte recovery obtained using CCE, immunomagnetic bead adherence, an alternative separation algorithm, was applied to the liquid cultures. This approach yielded highly variable results (Table 2). Megakaryocyte purities ranged from 13% to 67% and recoveries ranged from 20% to >100%. Greater purities appeared to be obtained from those unseparated liquid cultures exhibiting the highest initial megakaryocyte frequencies. CCE resulted in a greater consistency in megakaryocyte purity and an easier subsequent analysis of cells by flow cytometry. Based on this experience, we chose CCE for routine megakaryocyte enrichment.

Size and maturation stage distributions were determined for CCE-enriched megakaryocytes on days 12 to 13 of culture (Table 3, Fig 2). Among experiments, the overall pattern of distribution of megakaryocytes by maturation stage remained relatively constant. A majority of the megakaryocytes were distributed in stages II and III: a mean of 35% were stage II and 50% were stage III. An average of 8% of the cells were stage I and 7% were stage IV. On cytocentrifuge preparations, liquid culture derived megakaryocytes were large, exhibiting a mean diameter of 30.2 ± 1.5 μm in three experiments. Megakaryocyte size increased with maturation stage ranging from 22 μm for stage I cells to 38 μm for stage IV cells (Table 3). The distribution histogram for cultured megakaryocyte diameter is presented in Fig 2. Modal megakaryocyte diameter was 30 μm and diameters ranged from 13.5 μm to 63 μm.

Megakaryocyte maturation stage distributions varied substantially with culture duration (Fig 3). On day 9, 54% of the cultured megakaryocytes were stage II and 24% were stage III. By day 14 the inverse was observed; 63% of the cells were
Fig 4. Electron micrographs of liquid culture derived megakaryocytes grown from peripheral blood CFU-Meg. For the cell illustrated in A and B, immaturity is indicated by the presence of nucleoli (N), the large number of polyribosomes and the limited number of alpha granules. A demonstrates the multilobulated cell nucleus, diluted and normal demarcation membrane (DM), and well-preserved mitochondria (M). B is a fourfold magnification of a portion of the megakaryocyte in A (denoted by the rectangle). Normal demarcation membrane, an alpha granule (AG), golgi apparatus (G), polyribosomes (PR), and an autophagosome (AP) are identified. C illustrates a more mature liquid culture derived megakaryocyte. The nucleus is more highly lobulated, the nuclear:cytoplasmic ratio is decreased and demarcation membrane (some of which is dilated) is more prominent. (Original magnifications: A, x 11,250; B, x 46,000; C, x 7,500.)
stage III and 25% were stage II. Although partial synchrony in megakaryocyte maturation was present at culture intervals between 9 and 16 days, all maturation stages were detected at each culture interval (Fig 3).

Ultrastructure of the culture-derived megakaryocytes was well-preserved and similar to that of wild type, bone marrow derived megakaryocytes. Multi-lobulated nuclei (occasionally with nucleoli), large numbers of mitochondriae, Golgi apparatus, α-granules, and demarcation membrane formation were all identified (Fig 4).

Surface membrane platelet glycoprotein expression and ploidy of the liquid culture derived megakaryocytes were evaluated using flow cytometry. Illustrated in Fig 5 is a double-labeled megakaryocyte preparation stained for flow cytometric analysis. Both surface membrane and nuclear labeling are evident. Megakaryocytes labeled positively with monoclonal antibodies against both GP Ib and the GP IIb/IIIa complex. Within individual experiments, the proportion of cells positive for GP IIb/IIIa by flow cytometry corresponded closely to the percentage of megakaryocytes determined on cytocentrifuge preparations by Wright-Giemsa staining. Figure 6 is a representative ploidy distribution histogram of CCE-enriched megakaryocytes from a single liquid culture experiment determined using flow cytometry. A modal ploidy value of 16N is observed with clearly evident 4N, 8N, 16N, and 32N peaks. The 2N control signal is derived from human peripheral blood lymphocytes that were stained and analyzed concurrently with the megakaryo-
cytes. In a series of three separate liquid culture experiments, megakaryocyte ploidy distributions were very consistent (Table 4). In all instances, modal megakaryocyte ploidy values were 16N and over 80% of the megakaryocytes exhibited ploidy values between 8N and 32N inclusive.

In selected experiments, megakaryocyte ploidy distributions were determined both before and after enrichment by CCE. For these analyses, megakaryocytes were identified by positive labeling for anti-platelet GP Iib/IIIa. Figure 7 illustrates that CCE separation resulted in a disproportionate enrichment of high ploidy megakaryocytes. Modal ploidy shifted from 4N in the unseparated culture to 16N in the CCE-enriched preparation. These data are consistent with the known strong dependance of the CCE separation procedure on cell size.

Table 4. Ploidy Distribution Histograms of Liquid Culture Derived Megakaryocytes Enriched by Counterflow Centrifugal Elutriation and Analyzed by Flow Cytometry

<table>
<thead>
<tr>
<th>Experiment</th>
<th>4N</th>
<th>8N</th>
<th>16N</th>
<th>32N</th>
<th>64N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.9</td>
<td>23.7</td>
<td>49.1</td>
<td>17.6</td>
<td>2.7</td>
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<tr>
<td>2</td>
<td>8.0</td>
<td>15.9</td>
<td>49.1</td>
<td>23.1</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>15.1</td>
<td>33.2</td>
<td>44.2</td>
<td>6.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>10.0 ± 4.5</td>
<td>24.3 ± 8.7</td>
<td>47.5 ± 2.8</td>
<td>15.8 ± 8.3</td>
<td>2.4 ± 1.6</td>
</tr>
</tbody>
</table>

*Numbers represent the percentages of megakaryocytes in each ploidy class. In all instances, megakaryocytes were positively identified by staining for platelet GP Iib/IIIa.

DISCUSSION

Heretofore, investigations of the regulatory control system governing megakaryocytopenesis have proceeded almost independently of studies focusing on megakaryocyte biochemistry and cell biology. The former has been greatly facilitated by the development of methods for in vitro culture of the megakaryocyte progenitor cell, functionally defined as the CFU-Meg.25 Studies of the latter type have depended on increasingly effective cell separation algorithms applied to intact bone marrow.10,11 Only in the instance of megakaryocyte ploidy have perturbations affecting thrombopoietic regulation been linked to changes in cell biology.26-28 The recent identification and cloning of hematopoietic growth factors active in megakaryocytopenesis39,30 and an increasing recognition of the influence of megakaryocyte development on platelet heterogeneity and function11 emphasize the importance of integrating these two areas of research.

In the current investigation, we present an approach that permits such an integration. Megakaryocytes that develop in liquid culture are likely to be derived entirely from progeni-
Cyto growth in liquid culture requires the presence of a source of megakaryocytes, and discrepancies may be due to intrinsic differences between cultures is an example of such deviation from normal. These differences have been demonstrated in semisolid cultures, megakaryocyte growth in liquid culture requires the presence of a source of Meg-CSA. Thus, the entire megakaryocytic developmental process including mitotic cell expansion, polyploidization, and cytoplasmic maturation occurs in an in vitro liquid environment that is growth factor driven and amenable to controlled manipulation. Enriched, culture-derived megakaryocytes should thus provide opportunities to study the effects of growth factor regulation on megakaryocyte cell biology.

However, megakaryocytes developing in a liquid culture cannot be considered absolutely identical to those that are isolated from normal human bone marrow. The left-shifted ploidy distribution of the megakaryocytes in unenriched cultures is an example of such deviation from normal. These discrepancies may be due to intrinsic differences between peripheral blood and bone marrow megakaryocyte progenitors or more likely are due to the absence of required cytokine or stromal cell interactions in the liquid culture environment.

Both the apparent loss of low ploidy megakaryocytes and the relatively low megakaryocyte recovery following CCE enrichment also warrant comment. Small megakaryocytes are known to exhibit low ploidy and high density. Because cell sedimentation in the CCE chamber varies directly with both cell density and cell diameter squared, it is not surprising that small, relatively dense, low ploidy megakaryocytes are lost disproportionately. This loss probably accounts for the relatively large mean diameter of 22.2 μm that we observed for enriched stage I megakaryocytes. Our megakaryocyte recovery following CCE (averaging only 13%), is significantly lower than that previously reported by others. Shoff and Levine and Berkow et al. have achieved megakaryocyte recoveries of 93% and 58%, respectively, using human bone marrow aspirates. We believe our relatively low recovery is inherent to the liquid culture system. It may result from the disproportionate percentage of small, low ploidy megakaryocytes in the unseparated cultures as well as from the excessive stickiness and fragility of liquid cultured cells. The variability in enrichment obtained using immunomagnetic adherence is also probably attributable to excessive cultured cell adhesiveness. Using immunomagnetic beads, nonspecific cell clumping appears to result in substantial "trapping" of non-megakaryocytes in the bead/megakaryocyte rosettes. Of note, however, is that the average 77% recovery we achieved with immunomagnetic adherence compares favorably with the 36% recovery reported by Tanaka et al.

Unseparated megakaryocytes in liquid culture appear to be of lower ploidy and potentially are less mature. However, following CCE, the enriched megakaryocyte population exhibits stage and size distributions close to those previously reported for megakaryocytes isolated directly from normal bone marrow. Cultured megakaryocytes also express platelet GP Ib, GP IIb/IIIa, and CCE-enriched cells manifest DNA distributions that are similar to those exhibited by the latter set of cells. Therefore, one may anticipate that the biology of CCE-enriched, culture-derived megakaryocytes will be comparable with that of their wild type, bone marrow counterparts.

The megakaryocyte purity and viability that are achievable...
able using cells grown in liquid culture are similar to those reported from intact human marrow cells (Table 5). Significantly, however, the megakaryocyte yields from liquid culture are achieved using a starting sample of only 100 mL of normal peripheral blood. Comparable megakaryocyte yields from normal human marrow require whole human ribs or large volume bone marrow aspirates as the starting materials (Table 5). In addition, the numbers of liquid cultured megakaryocytes may also be conveniently scaled up by using either larger peripheral blood donations or mononuclear cells harvested by leukopheresis for the culture inoculum.

This investigation establishes a new approach for obtaining relatively large populations of normal human megakaryocytes, megakaryocytes of moderately high purity generated in vitro under controlled conditions. This experimental algorithm should be a valuable tool with which to explore the effects of growth factor regulation on human megakaryocyte phenotype, biochemistry, and function.

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Isolation of large numbers of enriched human megakaryocytes from liquid cultures of normal peripheral blood progenitor cells

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