Purification of Human Megakaryocytes by Fluorescence-Activated Cell Sorting

By Aaron Tomer, Laurence A. Harker, and Samuel A. Burstein

For direct studies of growth control, a method was developed to purify viable human megakaryocytes to homogeneity from routine normal bone marrow aspirates. An initial separation of marrow over a 1.050 g/mL Percoll density cut was used to enrich megakaryocytes. After washing, the cells were specifically labeled with a fluoresceinated monoclonal antibody or F(ab')2 fragment to the platelet glycoprotein (GP) IIb/IIIa complex. Megakaryocytes were selectively sorted by using Becton Dickinson FACStar flow cytometer on the basis of a fluorescence intensity >50-fold that of control cells. To increase resolution and purity the sorting rate was adjusted to one cell in 13 formed drops, and negative events that coincided with positive ones were aborted. Two thirds of the isolated cells were large, morphologically recognizable megakaryocytes with a forward light scatter fourfold that of the main cell population. Microscopic examination showed these cells to be ≥98% megakaryocytes with a diameter of 20 to 46 μm and a ploidy range of 2N to 64N with a mode of 16N. The small highly fluorescent cells were 10 to 21 μm in diameter, and their ploidy range from 2N to 32N with main ploidy classes of 2N and 4N. The majority of these small cells also positively reacted with monoclonal antibody to platelet GPIb. The isolated cells were cultured in either Iscove’s or leucine, lysine-deficient RPMI 1640 medium with 10% human plasma. The cells were maintained in culture more than three days and were capable of synthesis of both DNA and protein as assessed by radiolabeled thymidine and amino acid incorporation. Moreover, the isolated megakaryocytes were capable of responding to recombinant granulocyte-macrophage colony-stimulating factor. The data show that human megakaryocytes can be purified from routine marrow aspirates on the basis of a lineage marker and that they are capable of growth in vitro.

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STUDY OF THE BIOLOGY of megakaryocytes has proved difficult, in part due to their rarity (constituting about 0.05% of all nucleated bone marrow cells), fragility, and their tendency to aggregate.1-3 Thus, a large number of marrow cells and a highly selective method for megakaryocytic identification are needed for their isolation or analysis. Consequently, most studies of isolated megakaryocytes have used marrow flushed or scraped from the bones of experimental animals to achieve high cell yield with little admixture with blood and activated coagulation factors.4,5 For studies of human megakaryocytes this source is limited to surgical amputations.6,7

Both human and animal megakaryocytes have been isolated with variable results by using velocity centrifugation, which separates cells based primarily on their size, and density gradient centrifugation.1-3,5-14,17 Using marrow from human ribs Rabellino et al14 and Sitar18 obtained recoveries of 63% to 89% and purities of 68% to 94%. Notwithstanding these encouraging results, this method is limited by the need for ribs and the lack of specific megakaryocytic markers independent of size, which might result in a preferential selection of cells within the megakaryocytic population.2,11

Counterflow centrifugal elutriation (CCE) is an alternative approach. This procedure is based on the differences in sedimentation velocity between megakaryocytes and smaller cells.2,3,18 Enrichment by CCE results in 60% to 70% recovery and about 25% purity, which may be increased up to 50% to 65% when combined with density centrifugation.12,13 This method also inherently selects for larger megakaryocytes.

To overcome some of these technical limitations, flow cytometry has been introduced for the analysis of megakaryocytes.5,8,11 This method has been shown to be sensitive and rapid for the analysis of infrequent cell populations, even in complex cell mixtures such as exists in bone marrow.20 To prepare highly pure, viable human megakaryocytes isolated on the basis of a lineage marker on a routine basis, we have developed a method using fluorescence-activated cell sorting of normal bone marrow aspirates after labeling with a monoclonal antibody to the glycoprotein IIb/IIIa (GPIIb/
quently with medium containing citrate, adenosine, and theophylline in Hank's buffer (CATCH). This resulted in frequent cell aggregation in the Percoll gradient and cell clumping in the subsequent washing step. To reduce the likelihood of cellular aggregation, the heparin was replaced with ACD-A solution containing 2.5 mmol/L EDTA and 2.2 μmol/L PGE₃ (which was also used to rinse the aspiration needle). When 10 μmol/L of the synthetic thrombin inhibitor, t-phenylalanyl-l-propyl-l-arginine-l-chloromethylketone was added to the aspiration mixture, fibrin clot formation was reduced, but at the expense of increased cellular aggregation within the Percoll gradient. The dilution medium was supplemented with 3% BSA and initially with 2.5 mmol/L EDTA. This concentration of EDTA was subsequently found to be unnecessary, as was the addition of DNase. Indeed, it is unlikely that this divalent cation¬requiring nuclease is active in citrate- and/or EDTA-containing buffers. To improve viability, the theophylline concentration was reduced from 2 μmol/L (the original concentration in CATCH medium) to 1 mmol/L, and the adenosine component of CATCH medium was omitted with no observable increase in aggregation tendency.

Cell count, viability, and size measurement. Marrow cells were enumerated with a Hematology Series 15 Cell Counter (Baker Instruments, Allentown, PA) or a hemocytometer. Viability was assessed by trypan blue exclusion. The diameters of megakaryocytes freshly isolated by cell sorting (see the following sections) were measured with an ocular micrometer as previously described.

Fractionation of marrow cells. The cell suspension was adjusted to 20 x 10⁶ cells/mL, mixed with Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) to a final density of 1.020 g/mL (isotonic medium) to 20 x 10⁶/mL and admixture of the cells with Percoll to a final density of 1.020 g/mL (before layering over the 1.050 g/mL Percoll) was found to be helpful in preventing aggregate formation during centrifugation, presumably because the cells were dispersed in a larger volume rather than being concentrated at the interface. To prevent cell clumping during the successive washing step, the concentration of BSA was increased to 6%.

Megakaryocyte labeling. Megakaryocytes were directly labeled with saturating concentrations of fluoresceinated IgG1 mouse monoclonal antibodies (or F(ab)₂ fragment thereof) directed to the platelet GPIb/IIIa epitope. The antibodies reacted only with platelets and megakaryocytes. These antibodies (designated P₄ and P₅) were a gift from Dr Z.M. Ruggeri of the Research Institute of Scripps Clinic and were fluoresceinated to an F/P ratio of 3:1 by standard techniques. The cell count was adjusted to 10⁷/mL with MK medium containing 6% BSA and then incubated with 8 μg/mL antibody for 30 minutes on ice. Dead cells as well as unbound antibody were removed by centrifugation at 100 g for seven minutes at 15°C over 10% BSA. The cell pellet was then resuspended with MK medium containing 10 mmol/L HEPES buffer and 50 U/mL penicillin-streptomycin solution. The saturating concentration of the monoclonal antibodies was determined by quantitative flow cytometric measurements of the fluorescence of large megakaryocytes. An aliquot of the cell suspension was incubated under identical conditions with a fluoresceinated IgG1 mouse monoclonal antibody to human thyroglobulin and used as the control cells. The total time required to prepare the marrow cells for sorting was two to 2.5 hours.

Flow cytometry. Megakaryocytes were sorted with a FACStar flow cytometer (Becton Dickinson, Mountainview, CA) equipped with a 2 W Coherent, Innova 70 argon ion laser and a 120-μm-diameter nozzle. The sheath pressure was adjusted to the minimum level allowing stable drop formation (6.2 psi), with a sample pressure of 2 psi. The drop formation frequency was set to about 13.1 kHz, and the cell concentration was adjusted to allow sorting of no greater than 1,000 events/s. The number of drops collected for a single positive event was set to three to improve the yield without compromising the purity because only one of 13 drops contained detected particles. Purity was also enhanced by aborting negative events coinciding with positive ones. The stability of the fluid jet and the drop delay setting were monitored frequently. Small particles up to 7 μm were electronically gated out on the basis of forward-angle light scatter (FSC) by using 5.3-, 7.8-, and 10.2-μm standard polystyrene beads (Becton Dickinson), human RBCs, and peripheral blood lymphocytes. The laser emission was adjusted to deliver 200 mW at 488 nm for excitation of the flow cytometer. A 570-nm dichroic mirror was used to separate the fluorescence emissions of the fluorescein (green) and the propidium iodide (red) when it was used for concurrent ploidy measurement (see the next section). The green fluorescence was further filtered by a 530/30-nm band-pass filter, and red fluorescence was passed through a 625/35-nm band-pass filter before detection by the photomultipliers.

Megakaryocytes were identified by their distinct high green fluorescence (>50-fold that of the main cell population or that of the control cells). Megakaryocytes formed a discrete subset when the fluorescence emission was plotted against FSC (either by dot plot or contour histogram). Small megakaryocytes were defined by FSC levels corresponding to cell diameters up to 20 μm. The levels of fluorescence intensity, FSC, and side scatter of each cell were analyzed with a Consort 30 computer program (Becton Dickinson). When cells were collected for culture, the fluidic system was first sterilized with 70% ethanol followed by sterile PBS, and the cells were sorted into MK medium. The sample tubes were kept on ice and the collecting tubes were kept at 4°C. The adjustment of the flow cytometer required about one hour when experience was achieved. Sorting of an average cell sample required about two hours.

Cell ploidy determination. Cell ploidy was determined by flow cytometric measurement of the relative cell DNA content after staining by propidium iodide in hypotonic citrate solution as described by Krishan. Immunoenzymatic staining for GPIb.

Small cells, isolated on the basis of high fluorescence level only (and thus high expression of the epitope identified by P₄ or P₅), were examined for the additional
substance (kit I) for 20 minutes, washed with water, and counter-
stained with hematoxylin.

**Megakaryocyte culture.** Isolated marrow megakaryocytes were 
concentrated by centrifugation at 100 g for five minutes at 15°C and 
resuspended in 120 μL of MK medium. A diluted cell sample was 
counted, and the viability was assessed by trypan blue dye exclusion. 
Five hundred to 3,000 viable megakaryocytes were plated in 
duplicate microculture wells (Corning Glass Works, Corning, NY) 
containing 200 μL Iscove’s medium (Irvine Scientific, Irvine, CA) 
for studies of thymidine incorporation or leucine, lysine-deficient 
RPMI 1640 medium (Irvine) for studies of amino acid incorporation. 
Both media were supplemented with 10% dialyzed normal 
human plasma, 200 μmol/L L-glutamine, and 50 μmol/L 
2-mercaptopethanol. In some experiments the serum was replaced 
with 1% Nutrieye (J. Brooks Laboratories, San Diego) and 3% 
crystalline BSA (Sigma).

In subsequent experiments, cells were collected directly into tissue 
culture chambers mounted on plastic slides (Lab-Tek, Miles Scien-
tific, Naperville, IL) containing culture medium. Cultures were 
incubated in a humidified 5% CO2 tissue culture incubator at 37°C.

**Radioisotope incorporation.** To investigate the capacity of 
megakaryocytes to synthesize DNA and protein, cultures were 
radioactively labeled with 2 μCi of either [3H]thymidine ([3H]tdR, 87 Ci/mmol) or a 
[3H]-leucine-lysine mixture (120 Ci/mmol and 77 Ci/mmol respec-
tively; Amersham Corp. Arlington Heights, IL). Similar amounts of 
radioisotopes were added to control wells containing no cells. After 
20 hours in culture, the cells were harvested into 10% cold trichloro-
aetic acid (TCA). TCA-precipitable material was counted with a liquid 
scintillation counter (model LS 7000, Beckman Instruments, Fullerton, 
CA). In some experiments aminopterin and hypoxanthine 
(4 x 10-7 mol/L and 1 x 10-4 mol/L final concentrations, respec-
tively) were added to the culture medium to enhance the incorpora-
tion of thymidine. The significance of differences in incorporation 
rates was assessed with Student’s t test.

**Granulocyte-macrophage colony-stimulating factor (GM-
CSF).** To assess the response of isolated megakaryocytes to hema-
topoietic growth factors, sorted megakaryocytes were cultured in the presence of human recombinant GM-CSF, a gift from Dr Ken 
Kaushansky, University of Washington, Seattle. The factor was 
high-performance liquid chromatography-purified from serum-free medium conditioned by COS monkey kidney cells transfected with 
human GM-CSF cDNA. The final preparation was about 70% pure, 
and its specific activity as assayed on human bone marrow was 
approximately 1 x 104 U/mL. The preparation was diluted to 25 
U/mL with 2% human platelet-poor plasma in PBS.

**RESULTS**

**Cell separation.** As shown in Table 1, the yield of 
nucleated bone marrow cells from 6 to 8 mL of aspirated 
marrow was 434 ± 280 x 10^6 (n = 18). It was estimated that 
morphologically identifiable megakaryocytes constituted about 0.05% of this cell fraction. The Percoll density gradient 
fractionation resulted in the depletion of 97.5% ± 0.5% of the total 
marrow cells and an increase in megakaryocytic frequency to 2.1 ± 0.4, which represents a 40-fold enrichment. The light-density 
≤ 0.1050 g/mL fraction contained >90% of morphologically recognizable megakaryocytes.

After the labeling procedure with the antibody and two washing 
steps, the yield of the cells initially recovered from the light-density fraction was 50% (the yield of cells from the 
dense Percoll fraction >0.1050 g/mL was 70%). Cell viability 
before sorting was about 90%.

**Selection of GPIIb/IIIa-positive cells.** Figure 1 demon-
strates the fluorescence distribution of megakaryocyte-
reinforced bone marrow labeled with the GPIIb/IIIa-specific 
monoclonal antibody (A, the intact antibody; B, the F(ab')2 
fragment) compared with the same sample labeled with the 
control monoclonal antibody to human thyroglobulin (C). A 
peak of highly fluorescent cells was detected in the sample 
labeled with the specific probe. These cells constitute about 
2.5% of the total cell population (similar to the percentage of 
megakaryocytes estimated on this cell preparation using 
morphologic criteria) and exhibit a fluorescence intensity of 
50-180-fold that of the major cell population (the higher 
level being observed with the F(ab')2 fragment, B).

To improve megakaryocytic identification and to set crite-
rria for reproducible selection, the fluorescence intensity was 
plotted against FSC, a parameter reflecting, in large part, 
cell size (Fig 2). The bidimensional plot revealed a discrete 
subset with both high fluorescence intensity and an FSC 
fourfold that of the major cell population (Fig 2, upper right 
quadrant). After sorting and staining with hematoxylin, 
these cells appeared to be morphologically pure megakaryo-
cytes (Fig 3). Sorting and staining of the unselected marrow 
cell population (Fig 2, lower quadrants) revealed that the 
percentage of morphologically recognizable megakaryocytes 
in this fraction was between 2.5% and 5% of that found in the selected population.

The yield of megakaryocytes from an average marrow

<table>
<thead>
<tr>
<th>Table 1. Isolation of Megakaryocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleated Cells</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Unfractionated marrow</td>
</tr>
<tr>
<td>Percoll density cut (≤1.050 g/mL)</td>
</tr>
<tr>
<td>Presorting†</td>
</tr>
<tr>
<td>Postsorting‡</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*An estimate based on previous work.†The calculation is based on the mean of the nucleated cell number and the frequency of megakaryocytes in the preparation (n = 18).‡Cell suspension after labeling with monoclonal antibody and two washing steps.§Cells collected directly into chamber slides containing medium.
Table 1. Clinical and Laboratory Data of Patients Treated with Alphamethyldopa

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Months on AMD</th>
<th>Hematocrit H+/R+</th>
<th>Reticulocyte Count (%) H+/R+</th>
<th>Bilirubin (Total) H+/R+</th>
<th>Haptoglobin (mg/dl) H+/R+</th>
<th>Time Interval Between the Initial and the Remission Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70</td>
<td>F</td>
<td>3</td>
<td>28/42</td>
<td>25/0.6</td>
<td>2.4/0.3</td>
<td>25/110</td>
<td>3 mo</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>F</td>
<td>5</td>
<td>21/37</td>
<td>19/0.6</td>
<td>3.0/0.2</td>
<td>0/195</td>
<td>9 mo</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>F</td>
<td>24</td>
<td>24/38</td>
<td>12.5/0.1</td>
<td>2.9/N.D.</td>
<td>10/90</td>
<td>8 mo</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>F</td>
<td>24</td>
<td>20/37</td>
<td>20/0.1</td>
<td>3.0/0.2</td>
<td>30/170</td>
<td>21 mo</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>M</td>
<td>9</td>
<td>30/40</td>
<td>12/1.0</td>
<td>1.2/0.5</td>
<td>10/145</td>
<td>22 mo</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>M</td>
<td>48</td>
<td>25/44</td>
<td>18/0.2</td>
<td>3.7/N.D.</td>
<td>10/175</td>
<td>26 mo</td>
</tr>
<tr>
<td>7</td>
<td>78</td>
<td>F</td>
<td>36</td>
<td>24/39</td>
<td>18/1.5</td>
<td>2.4/0.41</td>
<td>0/115</td>
<td>7 mo</td>
</tr>
<tr>
<td>8</td>
<td>70</td>
<td>M</td>
<td>60</td>
<td>31/38</td>
<td>3.5/0.6</td>
<td>2.0/0.34</td>
<td>10/130</td>
<td>7 mo</td>
</tr>
<tr>
<td>9†</td>
<td>57</td>
<td>M</td>
<td>36</td>
<td>42</td>
<td>2</td>
<td>0.7</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>10†</td>
<td>66</td>
<td>F</td>
<td>48</td>
<td>42</td>
<td>0.7</td>
<td>0.1</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>11†</td>
<td>71</td>
<td>F</td>
<td>60</td>
<td>42</td>
<td>0.9</td>
<td>0.5</td>
<td>175</td>
<td></td>
</tr>
</tbody>
</table>

*During hemolysis.
†During recovery phase.
‡Nonhemolyzing DAT-positive.

IgG, and anti-C1q were further established by inhibition tests using purified IgM* and IgG immunoglobulins. Erythrocytes obtained from normal donors did not react with anti-IgM, anti-IgA, anti-C4, or anti-C1q antibodies (ΔOD < 0.05). As observed by other investigators123 reactions were detected variably with antibodies against C3, IgG, K, and λ chains; the "normal" erythrocyte-bound IgG being commonly IgG. All the antitglobulin reagents described in Table 1 were tested against erythrocytes from two patients who had reticulocytosis (18% and 20%) without immunologic diseases. A positive reaction was not obtained in the continuous flow test system excluding possible antitransferrin activity in the antitglobulin reagents. Stock solutions of antitglobulin reagents were kept at −20°C and dilutions were made in 0.5% BSA in 0.9% NaCl. The presence of erythrocyte antibodies in the patients’ sera was investigated by indirect AGT: normal ABO compatible erythrocytes, free of detectable IgG and C3, were washed and incubated with 4 vol of the undiluted patients' sera for 2.5 h at 24°C. The cells were then washed 5 times with 25 vol of 0.9% NaCl and tested in the PVP-AGT system for the presence of cell-bound immunoglobulins and complement. Similar experiments were carried out for determination of the immunoglobulin class of the antibodies present in eluates prepared from erythrocytes of some of the patients. The eluates were mixed with equal volumes of normal fresh serum to provide complement components, and the mixtures were incubated with erythrocytes for an additional 30 min at 37°C. These eluates had been prepared from patients' washed erythrocyte ghosts, using glycine buffer (pH3) as previously described.4

For the manual antitglobulin tests, a polyspecific antitglobulin reagent (Ortho Diagnostics, Raritan, N.J.) was used. The Polybrene test and the method for determination of cell-bound antibody titers have been previously described.9-12 Briefly, for cell-bound antibody titration10 aliquots from a 6.5% suspension of the patients' erythrocytes were mixed in various proportions with a 6.5% suspension of washed normal cells and the mixtures were run through the Polybrene flow system. The ΔOD recorded reflected the activity of the antibodies derived from the patients' erythrocytes. The effects of heat on antigen-antibody complexes were investigated by obtaining temperature gradient dissociation curves (TGDC) as described elsewhere.11 The patients' antibody-coated erythrocytes were washed and then run continuously through the flow system. The antibody-dependent aggregates were subjected to gradually increasing temperature from 30°C to 60°C and the OD changes.
estimated to be $15.2 \pm 4.8 \mu m$ ($n = 4,000$). Because the relationship between FSC and the size of large cells had not been established, the mean diameter of unfixed large cells was measured microscopically and averaged $34.2 \pm 11.8 \mu m$ ($n = 150$). Table 2 demonstrates the ploidy distribution of both the large and the small megakaryocytes as measured by propidium iodide staining. The modal ploidy of the large cells was 16 (40.6%), with 30.0% of cells being 32N, whereas about 60% of the small cells were 2N to 4N.

**Cell culture.** Megakaryocytes were cultured in liquid medium in microwell plates or slide chambers (Fig 4, culture of large cells). The viability, as assessed by trypan blue dye exclusion, ranged between 50% for the large cells and 75% for the small ones. Cells were maintained in culture more than three days. Twenty-four hours after the initiation of culture, only 50% to 70% of the cells appeared to be viable, showing a round shape, refractile cytoplasm, and nondiscernable nuclear outline.

To examine the biosynthetic ability of the isolated cells, incorporation of $^3$HThdR and a $^3$H-leucine-lysine mixture into TCA-precipitable material was measured. Thymidine incorporation was significantly higher by the small cells than by the large ones ($P < .05$; Table 3). The incorporation rate of all megakaryocytes was between that of the small and the large cells. The addition of hypoxanthine and aminopterin to the culture medium resulted in a six- to sevenfold increase in the incorporation rate by the large cells (data not shown). The effect was pronounced only in cultures that were treated with the factor for about 20 hours before pulse-labeling with the radioisotopes. No increase in cell number was observed during the culture period.

**DISCUSSION**

Purified viable human megakaryocytes are required to study some aspects of the biology of these cells such as biosynthesis of specific proteins or the response to growth factors. Based on recent studies demonstrating the utility of fluorescence-activated cell sorting for analysis of animal megakaryocytes,5,6,9-11 we have established a technique for

<table>
<thead>
<tr>
<th>Table 2. Ploidy Distribution of Small and Large Megakaryocytes</th>
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</thead>
<tbody>
<tr>
<td><strong>Megakaryocytes</strong></td>
</tr>
<tr>
<td>Small</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Large</td>
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<td></td>
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</table>

*Percentage of total.

**Table 3. $^3$HThdR Incorporation by Isolated Megakaryocytes**

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Cells</th>
<th>cpm</th>
<th>cpm/Cell (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% plasma Small</td>
<td>1,500</td>
<td>1,950 ± 282</td>
<td>1.3</td>
</tr>
<tr>
<td>10% plasma + AH* Small</td>
<td>1,500</td>
<td>13,842 ± 1,633</td>
<td>9.2</td>
</tr>
<tr>
<td>10% plasma + AH* Large</td>
<td>1,500</td>
<td>7,212 ± 1,175</td>
<td>4.8</td>
</tr>
<tr>
<td>All</td>
<td>650</td>
<td>4,225 ± 548</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*Aminopterin (A) was added to a final concentration of $4 \times 10^{-7}$ mol/L and hypoxanthine (H) to $1 \times 10^{-4}$ mol/L.

**Table 4. Amino Acid Incorporation by Isolated Megakaryocytes**

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Cells</th>
<th>cpm (± 1 SD)</th>
<th>cpm/Cell (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% plasma Small</td>
<td>2,000</td>
<td>15,040 ± 1,095</td>
<td>7.5</td>
</tr>
<tr>
<td>10% plasma Large</td>
<td>2,000</td>
<td>10,520 ± 1,237</td>
<td>5.2</td>
</tr>
<tr>
<td>All</td>
<td>2,000</td>
<td>12,020 ± 2,209</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Lysolemic, leucine-deficient RPMI 1640 medium supplemented with 10% dialyzed normal human plasma.

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Fig 4. Phase-contrast photomicrograph of sorted large megakaryocytes after 24 hours in culture (original magnification × 200; current magnification × 150).
the preparative sorting of human megakaryocytes. Using this method, we have shown that megakaryocytes (a) can be isolated on the basis of an intrinsic cell surface marker rather than physical characteristics, (b) can be characterized and separated further by size, (c) are biosynthetically intact with the small cells more active than the large cells, and (d) respond to at least one characterized hematopoietic growth factor, GM-CSF.

The method described herein presents several advantages. (a) Bone marrow aspirates are used as the starting material, thereby avoiding dependence on amputated bones and permitting serial studies to investigate cell physiology as well as clinical disorders of megakaryocytopoiesis. (b) The technique ensures a nearly pure and biosynthetically viable (50% separated further by size, (b) isolated on the basis of an intrinsic cell surface marker rather than physical characteristics, (c) are biosynthetically intact with the small cells more active than the large cells, and (d) respond to at least one characterized hematopoietic growth factor, GM-CSF.

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Table 5. Incorporation of 3H-TdR and 3H-Lysine-Leucine Into Megakaryocyte Cultures Treated With rGM-CSF

<table>
<thead>
<tr>
<th>GM-CSF Treatment</th>
<th>Time of Addition of Radiolabel</th>
<th>Cells Plated</th>
<th>Radiolabeled Precursor</th>
<th>cpm (± 1 SD)</th>
<th>3H-TdR</th>
<th>3H-Leu/Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>0 2,000 3H-TdR</td>
<td>18,000 ± 2,969</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>19,150 ± 2,725</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>1,500 3H-Leu/Lys</td>
<td>10,510 ± 1,334</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>9,820 ± 1,519</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>20 2,000 3H-TdR</td>
<td>15,420 ± 3,049</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>36,080 ± 6,500</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>1,500 3H-Leu/Lys</td>
<td>5,075 ± 681</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>7,830 ± 1,028</td>
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</tr>
</tbody>
</table>

Small megakaryocytes were cultured in 200 μL of either Iscove's medium plus AH for 3H-TdR incorporation or leucine-lysine-deficient RPMI 1640 medium for 3H-amino acid incorporation, both supplemented with 10% normal human plasma. Recombinant GM-CSF was added at 1 U/mL at the outset of culture. The radiolabeled precursors were added immediately or 20 hours after the addition of rGM-CSF. Eighteen hours later the cells were harvested, and TCA-precipitable material was counted as described. The data represent means (± 1 SD) of three experiments.
PURIFICATION OF HUMAN MEGAKARYOCYTES

...expression of GPIb by an immunoenzymatic staining technique. Approximately 75% of the small GPIIb/IIIa-positive cells also stained for GPIb. Based on data that GPIIb/IIIa appears or is detectable earlier in the differentiation sequence than GPIb, and on our previous data assessing the specificity of our anti-GPIIb/IIIa monoclonal antibodies,21,25 we conclude that these small cells expressing high levels of GPIIb/IIIa represent early cells in the megakaryocytic lineage that have yet to express the GPIb epitope.

As shown by us21 and others,11 the majority (70%) of small megakaryocytes are found at densities >1.050 g/mL. The choice of cells of density <1.050 g/mL in this study was dictated by the need for megakaryocyte-enriched marrow to establish the sorting method. Studies requiring greater numbers of small cells could be performed by increasing the Percoll density in the preliminary enrichment step. It is possible, however, to eliminate the enrichment procedure if sorting time is not a limitation.

The size range of megakaryocytes (10 to 46 μm) is similar to that reported by others,3,8,13 and the ploidy distribution is similar to that found in previous work21 except for the higher proportion of 2N and 4N cells, probably as a result of using a different technique.

Human megakaryocytes have the capacity to grow in culture and to be biosynthetically active.1,19 However, cultures of highly purified human megakaryocytes have not been examined. In the present selection method the isolated pure megakaryocytes were able to grow in medium supplemented with 10% plasma for at least three days and to show biosynthesis of proteins and DNA. An attempt to replace plasma by a defined medium was not fully successful, thereby indicating the inadequacy of the plasma substitute. Further studies will be required to determine whether this problem is trivial or related to the absence of a required growth factor(s). The low incorporation rate by the large cells suggests that mature megakaryocytes synthesize macromolecules at a lower rate than the small cells. However, it cannot be excluded that large cells were damaged during the isolation procedure to a greater extent than the small cells.13 The incorporation rate was decreased by 30% to 50% when cultures were pulse-labeled after 20 hours, which reflects, at least in part, the decrease in cell viability as a function of time in culture. A similar phenomenon was observed by Greenberg et al using CCE and density centrifugation to purify rat megakaryocytes.12

As has been found for other marrow cells, megakaryocytic proliferation and maturation are controlled by hematopoietic growth factors.9 Pure cultures of marrow-derived megakaryocytes provide the opportunity to study the direct effect of such factors on the maturation of these cells. In this study the effect of rGM-CSF was examined. The data showed that the small cells responded with a significant increase in thymidine and amino acid incorporation. The insignificant response of the large cells may reflect biologic nonresponsiveness of mature megakaryocytes or could be related to damage incurred during the isolation procedure. The response of the small cells became evident when the cells were precultivated for 20 hours with rGM-CSF. This delay may be due to a requirement for synthesis of a regulatory protein(s) before the onset of general protein synthesis or recovery of cellular responsiveness to GM-CSF because they were exposed to nonphysiological medium for several hours. It is conceivable that GM-CSF is stimulating the megakaryocyte colony-forming cell (CFU-MK) or possibly a myeloid progenitor cell is contaminating the cultures. Some investigators have suggested that CFU-MK express GPIIb/IIIa, which permits these cells to be sorted by our method.26 Mitigating against these possibilities are (a) the lack of an increment in cell number during the culture period, (b) the infrequency of progenitor cells (at least in mice) at Percoll densities <1.050 g/mL,13 and (c) more recent evidence suggesting that CFU-MK do not express GPIIb/IIIa.27 Nonetheless, GM-CSF appears to promote the biosynthesis of macromolecules in nonproliferating, presumably endoreduplicating megakaryocytes. This conclusion is not entirely surprising because GM-CSF has been shown to promote proliferation of progenitor cells of other lineages including the CFU-MK as well as to influence the functions of mature cells in the granulocytic lineage.24,30

Based on these results and the known analytic potential of fluorescence-activated cell sorting, we conclude that the present method provides a useful means for the isolation and analysis of human megakaryocytes. This technique may facilitate the study of normal megakaryocytopoiesis in humans as well as the investigation of various clinical disorders of thrombocytopoiesis.

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Purification of human megakaryocytes by fluorescence-activated cell sorting

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