Purification of Human Marrow Progenitor Cells and Demonstration of the Direct Action of Macrophage Colony-Stimulating Factor on Colony-Forming Unit–Macrophage

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To facilitate the investigation of the direct interaction between hematopoietic progenitors and colony-stimulating factors, we have developed a method to purify human marrow progenitor cells. Using density centrifugation, negative panning with concanavalin A coated plates, positive selection of CD34-positive cells with immunomagnetic microspheres, overnight adherence to a plastic dish, negative selection with a panel of monoclonal antibodies, and density centrifugation, human marrow progenitor cells were purified from 1.5% to 53.2%, a 42-fold purification, with a 4.8% yield. The purified cells consisted of 38% erythroid, 9% colony forming unit-granulocyte (CFU-G), 29% CFU-macrophage (CFU-M), 12% CFU-eosinophil/basophil (CFU-Eo/Ba), and 4% CFU-mix. The purified cells cultured in serum-free fibrin clots with recombinant human macrophage colony-stimulating factor (rM-CSF) for 14 days developed a pure population of CFU-M colonies. An appearance of CFU-M colonies was present after the addition of 1 U/mL of rM-CSF and the maximum stimulation was found at 100 U/mL. When the purified cells were cultured in serum-free medium with rM-CSF in a limiting dilution assay and the percentage of nonresponder wells for CFU-M colonies was plotted against cell concentration, serum-free cultures yielded a straight line through the origin, indicating that CFU-M development did not depend on accessory cells and that rM-CSF acted directly on the CFU-M.

MATERIALS AND METHODS

Cell separation by density centrifugation. Bone marrow samples were obtained from healthy adult volunteers with informed consent and 3 mL was collected in sodium heparin at a final concentration of 50 U/mL. The samples were well mixed with 5 mL of Dulbecco’s phosphate-buffered saline (PBS) containing 13.6 mmol/L sodium citrate, and the light-density mononuclear cells were separated over Ficoll-Hyphaque (FH; 1.077 g/cm³, Pharmacia Fine Chemicals, Piscataway, NJ) at 400g, 24°C, for 25 minutes. The interface mononuclear FH cells were collected and washed with Iscove’s modified Dulbecco’s medium (IMDM) containing 0.25% deionized bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO; Cohn fraction V, 96% to 99% pure) by centrifugation at 600g for 10 minutes at 24°C. The cell pellet was resuspended in 10 mL of IMDM containing 0.25% BSA and centrifuged at 600g for 5 minutes at 4°C and resuspended in IMDM containing 1% BSA at a concentration of 2 × 10⁶/mL (FH cells).

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Negative panning with concanavalin A-coated plates. Each 10 mL of FH cell suspension was incubated at 4°C for 45 minutes in 100-mm polystyrene tissue culture dishes (Corning; Iwaki Glass, Tokyo, Japan) that had been previously coated with concanavalin A (type IV; Sigma). The nonadherent cells were removed and centrifuged (Con A+ cells). The preparation of concanavalin A-coated plates was performed by the method of Wysocki and Sato.1 Poly styrene tissue culture dishes (100 x 20 mm) were incubated at room temperature with 10 mL of 0.05 mol/L Tris-HCl buffer, pH 9.5, containing 250 μg of concanavalin A. After 3 hours, the plates were washed three times in PBS, pH 7.4, once in PBS containing 0.1% BSA, and were then stored at -40°C until they were used.

Positive selection of cells coated with CD34. The positive selection procedure of CD34-positive (CD34+) cells was performed by the method described by Sawada et al with minor modifications. The Con A+ cells were resuspended in IMDM containing 1.0% BSA at a concentration of 3 x 10^7/mL, cooled to 4°C, and mixed with the same volume of CD34anti-My10 mouse hybridoma tissue culture supernatant at 4°C to coat lympho-hematopoietic progenitor cells.2,3 The cell suspension was incubated at 4°C for 30 minutes using an end-over-end rotator (Bel-art rotator, Model 71; TechniLab Instruments Inc, Pequannock, NJ). After 30 minutes of incubation, the cell suspension was transferred to a 15 mL conical tube (Becton Dickinson and Co, Lincoln Park, NJ) and 1.5 mL of 10% BSA was gently underlaid. The tube then was centrifuged at 400g for 5 minutes at 4°C. The anti-My10 hybridoma supernatant and approximately 0.5 mL of 10% BSA were removed, leaving 1 mL of 10% BSA in the tube. The tube wall then was washed once by gentle application of 5 mL of IMDM containing 0.25% BSA, while the rest of the 10% BSA over the cell pellet was used to protect the cells from the washing procedure (cell protection washing procedure). After washing the tube wall, the 10% BSA over the cell pellet was removed, and the cells were washed once with cold IMDM containing 0.25% BSA. The cells were resuspended in IMDM containing 1.0% BSA at a concentration of 3 x 10^7/mL in a 12 x 75 mm polystyrene tube (Becton Dickinson) and were incubated with immunomagnetic microspheres (Dynabeads M450 coated with goat antimouse IgG; Dynal Inc, Great Neck, NY), with a microsphere per cell ratio of 3:1, at 4°C using an end-over-end rotator. After 50 minutes of incubation, the cell-bound microspheres and free microspheres were attached to the tube wall by a magnet (Dynal MPC-1; Dynal Inc, Fort Lee, NJ), and the free cells that did not bind microspheres were removed by washing the tube three times with 3 mL of cooled IMDM containing 0.25% BSA. The tube was then disconnected from the magnet and the microspheres were washed by gentle pipetting with 3 mL of cooled IMDM to remove BSA. The microspheres were again attached to the tube wall using the magnet, and cells released by washing were removed. The tube wall was washed once with cooled IMDM. The cells that bound microspheres and free microspheres were gently pipetted for 30 seconds with 1.5 mL of IMDM containing 130 U/mL of chymopapain (Chymodiactin; Boots Company Inc, Lincolnshire, IL) and 0.02 mol/L disodium ethylenediamine tetraacetate (EDTA; Wako Chemicals, Osaka, Japan) at 37°C. The tube was attached to the magnet for 1 minute at 24°C. The cells released from the microspheres were collected and designated as CD34+ fraction (Fr 1). The CD34+ Fr 1 was immediately mixed with 4.5 mL of heat-inactivated fetal calf serum (FCS; Flow Laboratories, Inc, McLean, VA) at 24°C in a 15-mL conical tube to dilute the chymopapain activity. This separation procedure was sequentially repeated another two times to collect CD34+ Fr 1 to 3 and the cells were pooled in the same tube containing FCS. After the cell protection washing procedure was performed, the cells were collected and resuspended in IMDM containing 0.25% BSA (CD34+ cells).

Adherent cell depletion. The CD34+ cells were incubated overnight, at 37°C, in 5 mL IMDM containing 20% FCS, 10% heat-inactivated pooled human AB serum, 1% BSA, 50 U/mL of recombinant human interleukin-3 (rIL-3) (10 U/mL AmGen Biologicals, Thousand Oaks, CA), 50 U/mL of recombinant human granulocyte-macrophage colony-stimulating factor (rGM-CSF) (4.5 x 10^7 U/mL; Schering Research, Bloomfield, NJ), 50 U/mL of recombinant human granulocyte-CSF (rG-CSF) (2.5 x 10^7 U/mL; Chugai Pharmaceutical Co, Tokyo, Japan), 2 U/mL of recombinant human erythropoietin (rEP) (146,000 U/mL; Snow Brand Co, Tokyo, Japan), 10 μg/mL insulin (porcine sodium, activity 26.3 USP U/mg; Calbiochem, Behring Diagnostics, La Jolla, CA), 5 x 10^7 mol/L 2-mercaptoethanol (2-ME; Sigma), penicillin at 50 UI/mL, and streptomycin at 50 μg/mL (Flow Laboratories) in a 25-cm² tissue culture polystyrene flask (Becton Dickinson) placed into a 5% CO₂/95% atmosphere incubator. The nonadherent day 1 cells were removed with gentle washes and centrifuged (AD- cells).

Antibody-coated cell depletion by negative selection. The AD- cells were suspended in 0.84 mL IMDM containing 1.0% BSA in a 2-mL tube (Sarstedt, Rommelsdorf, Germany), cooled to 3°C, and the mouse MoAbs listed in Table 1 were added to coat granulocytes, monocytes, natural killer cells, T or B lymphocytes, at the final concentrations shown.11,12 The final volume of the cell suspension was 1 mL, which was incubated for 30 minutes at 3°C with end-over-end rotation before being transferred to a 15-mL conical tube for the cell protection washing procedure. After removal of the rest of the 10% BSA, the cells were washed once with 10 mL IMDM containing 0.25% BSA at 4°C. The cells were resuspended in 2 mL IMDM containing 1.0% BSA and then were mixed with immunomagnetic microspheres coated with goat antimouse IgG with a ratio of 40:1 in a 12 x 75 mm polystyrene tube (Becton Dickinson). The tube was incubated with an end-over-end rotator at 3°C. After 60 minutes, the cells that did not bind microspheres were collected by magnetic separation (AB- cells).

Removal of dead cells. The AB- cells were resuspended in 2 mL IMDM containing 0.25% BSA and were overlaid on FH in a 12 x 75 mm polystyrene tube. After centrifugation at 600g with rapid

Table 1. Mouse MoAbs Used for Negative Panning

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Ig Concentration</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>CD2 anti-OKT*11</td>
<td>0.2 μg/mL</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>CD3 anti-OKT*3</td>
<td>0.06 μg/mL</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>CD7 anti-Leu8</td>
<td>0.1 μg/mL</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>CD10 anti-OKBcALL</td>
<td>0.2 μg/mL</td>
<td>B lymphocytes, granulocytes</td>
</tr>
<tr>
<td>CD11b anti-OKM*1</td>
<td>0.4 μg/mL</td>
<td>Monocytes, granulocytes, natural killer cells</td>
</tr>
<tr>
<td>CD16 anti-My23</td>
<td>20 μL/mL</td>
<td>Mature granulocytes, monocytes</td>
</tr>
<tr>
<td>CD19 anti-Leu12</td>
<td>0.1 μg/mL</td>
<td>B lymphocytes</td>
</tr>
<tr>
<td>CD20 anti-Leu16</td>
<td>0.1 μg/mL</td>
<td>B lymphocytes</td>
</tr>
</tbody>
</table>

Mouse hybridoma tissue culture supernatants are given as the final volume in 1 mL of cell suspension, or the final Ig concentration.
acceleration for 5 minutes at 24°C, the interface mononuclear cells (AB- FH cells) were collected into a 15-mL conical tube for the cell protection washing procedure. The rest of the 10% BSA then was removed, and the cells were resuspended in IMDM containing 0.25% BSA to be kept at 3°C until use. The viability of these cells was measured by dye exclusion using 0.2% trypan blue dye.18

Culture procedure for progenitor cells. Aliquots of cells from each fraction were cultured in triplicate at various concentrations ranging from 5 x 105 to 2 x 106 cells/mL in 24-well flat-bottomed tissue culture plates (Linbro; Flow Laboratories) with a 0.5 mL mixture containing 20% FCS, 10% pooled human AB serum, 1% BSA, 5 x 10−4 mol/L 2-ME, 1.5 mmol/L e-aminocaproic acid (EACA; Kishida Chemical Co, Osaka, Japan), penicillin-streptomycin, 10 μg/mL insulin, rEP at 2 U/mL, rIL-3 at 50 U/mL, rGM-CSF at 50 U/mL, rG-CSF at 50 U/mL, 2 mg/mL human fibrinogen (grade L, coagulability 90% of total protein content; KabiVitrum, Stockholm, Sweden), 0.2 U/mL thrombin (Parke-Davis, Morris Plains, NJ), and IMDM. For serum-free culture, FCS and pooled human AB serum were substituted by 300 μg/mL of iron-saturated transferrin (purity 98%; Sigma) and lipid suspension (oleic acid, 2.8 μg/mL; L-a-phosphatidylcholine, 4.0 μg/mL; cholesterol, 3.9 μg/mL; Sigma) as described previously. After 14 days of incubation at 37°C in a 5% CO2/95% atmosphere, the clots were fixed and stained. rM-CSF (2.1 x 106 U/mL) was a gift of Genetics Institute (Cambridge, MA). After benzidine-hematoxylin staining, enumeration of burst-forming unit-erythroid (BFU-E) was performed in accordance with the criteria of Clark and Houssain.19 The colonies that gave rise to 8 to 49 erythroblasts after 14 days of culture were counted as nonerythroid colonies, while the colonies that gave rise to 40 or more cells were counted as erythroid colonies. The colonies that gave rise to 40 or more nonhemoglobinized cells were counted as erythroid-mix, BFU-E, and nonerythroid colonies, while the colonies that gave rise to 40 or 20 macrophages were counted as erythroid-mix colonies. The colonies that gave rise to 10 to 19 macrophages were counted as erythroid-mix clusters.

RESULTS

Purification of hematopoietic progenitor cells from human marrow. Table 2 shows the mean yield and enrichment of human marrow progenitor cells in seven experiments when FH mononuclear cells were sequentially purified by negative panning with concanavalin A-coated plates (Con A−), positive selection of the cells bearing the CD34 surface marker (CD34+), depletion of adherent cells (AD−), negative selection of the cells bearing surface markers for the MoAbs listed in Table 1 (AB−), and the depletion of dead cells (AB-FH). To determine the number of progenitor cells, each fraction was cultured in serum-containing fibrin clots for 14 days. Through these procedures, human marrow progenitor cells were purified from 1.5% ± 0.7% (FH) to 53.2% ± 6.51% (AB-FH), with a range of 45% to 67%, a 42-fold purification with a 4.8% yield and 97% ± 3% viability. When AB-FH cells were cultured for 7 days with rEP, they promoted BFU-erythroid (BFU-E) that gave rise to 8 to 49 cells (data not shown). However, in this report, we did not add the purity of CFU-E in Table 2 because of the difficulty in distinguishing CFU-E that gradually degraded and declined in the clots from small erythroid colonies that were retained for 14 days in the clots. By light microscopy, AB-FH cells consisted predominantly of very immature blast cells (Fig 1A).

This purification method is characterized by positive selection of CD34+ cells using immunomagnetic microspheres coated with goat antimouse IgG and the release of microspheres from CD34+ cells for further purification steps. A representative experiment is shown in Fig 2A. In the CD34+ Fr 1, 68% of the progenitors in the Con A− cell fraction were released; however, 93% of released progenitor cells gave rise to small erythroid colonies. The CD34+ Fr 5 was pipetted with IMDM to remove BSA and, as a result, 11% of the progenitors in the Con A− cells were released, but 58% of the released progenitor cells gave rise to small erythroid colonies. The mixture was then treated with chymopapain to release the cells from the microspheres. CD34+ Fr 1 to 3 contained 12% of the total progenitor cells, including small erythroid colony progenitors in the Con A− cells and 52% of erythroid-mix, BFU-E, and nonerythroid progenitor cells in the Con A− cells.

### Table 2. Purification of Human Marrow Progenitor Cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Cells</th>
<th>Purity of Progenitors (%)</th>
<th>Purification Factor (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>Viability (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FH</td>
<td>516 ± 391</td>
<td>100</td>
<td>97 ± 2</td>
<td>1.51 ± 0.71</td>
</tr>
<tr>
<td>Con A−</td>
<td>309 ± 209</td>
<td>63.7 ± 14.4</td>
<td>96 ± 2</td>
<td>2.32 ± 0.78</td>
</tr>
<tr>
<td>CD34+</td>
<td>3.84 ± 3.03</td>
<td>0.83 ± 0.68</td>
<td>99 ± 1</td>
<td>22.3 ± 5.82</td>
</tr>
<tr>
<td>AD−</td>
<td>2.62 ± 1.96</td>
<td>0.59 ± 0.48</td>
<td>95 ± 4</td>
<td>25.8 ± 5.61</td>
</tr>
<tr>
<td>AB−</td>
<td>1.18 ± 1.04</td>
<td>0.27 ± 0.26</td>
<td>87 ± 7</td>
<td>39.9 ± 12.2</td>
</tr>
<tr>
<td>AB-FH</td>
<td>0.71 ± 0.68</td>
<td>0.16 ± 0.15</td>
<td>97 ± 3</td>
<td>53.2 ± 6.51</td>
</tr>
</tbody>
</table>

Human marrow progenitor cells were purified from 3 mL blood using density centrifugation (FH), negative panning with concanavalin A-coated plates (Con A−), positive selection with CD34/anti-My10 MoAb (CD34+), adherent cell depletion (AD−), negative selection with MoAbs listed in Table 1 (AB−), and density centrifugation (AB-FH). The cells from each fraction were cultured in triplicate in 0.5 mL fibrin clots at a range of concentrations of 50 to 20,000 cells/mL with 2 U/mL of rEP, 50 U/mL of rIL-3, 50 U/mL of rGM-CSF, and 50 U/mL of rG-CSF. Mean values for seven experiments ± SD are shown.
The effect of negative selection and the yield and purity of the progenitor cells in each fraction are shown in Fig 2B. The AB$^-$ Fr 1 that had not been suspended by gentle pipetting contained 74% of the progenitor cells with a purity of 52%. The purity of the progenitor cells was dramatically decreased in the AB$^-$ Fr 2 after gentle pipetting. Therefore, in the negative panning procedure, only AB$^-$ Fr 1 was collected and used.

**Lineage of progenitor cells in purified cells.** To investigate the lineage of the progenitor cells in the purified cells, AB$^{-}$ FH cells were cultured with rIL-3, rGM-CSF, rG-CSF, and rEP in serum-containing medium, and with rIL-3, rGM-CSF, rG-CSF, rM-CSF, and rEP in serum-free medium, as shown in Table 3. The progenitor cells in the AB$^-$ FH cells contained cells that gave rise to 1% erythroid-mix, 29% BFU-E, 16% small erythroid, 3% nonerythroid-mix, 9% CFU-G, 29% CFU-M, and 12% CFU-Eo/Ba colonies. When rM-CSF was added to the serum-free medium, the AB$^-$ FH cells expressed the same number of colonies with the same lineage expression as those observed in serum-
M-CSF acts directly on human CFU-M

were collected after gentle pipetting.

The microsphere-cell mixture was then incubated with chymopapain. Cells that detached from the microspheres were collected using the magnet and designated as CD34+ fractions. This procedure was repeated four times. CD34+ Fr 1 through 4 were pooled and incubated overnight to remove adherent cells. The nonadherent cells (AD) contained medium. By astra blue-eosin staining, two types of eosinophil colonies were observed. One group consisted of eosinophils and basophils. The other group consisted of cells stained by both astra blue and cosin (data not shown).

The microsphere-cell mixture was then suspended by gentle pipetting to disperse the aggregations of microspheres and cells; CD34+ Fr 2 through 4 and 6 were the cells collected by washing the tube wall while the magnet was applied. The microsphere-cell mixture was then incubated with chymopapain. Cells that detached from the microspheres were collected using the magnet and designated as CD34+ fractions. This procedure was repeated four times. CD34+ Fr 1 through 4 were pooled and incubated overnight to remove adherent cells. The nonadherent cells (AD) containing 2.85 \times 10^6 total cells with 20.3% progenitor cells were then incubated with a panel of MoAbs listed in Table 1 to coat contaminant cells.

The purified cells (AB FH) were cultured in triplicate in 0.5 mL of medium at a concentration of 200 cells/mL in the presence of 50 U/mL of rIL-3, 50 U/mL of rGM-CSF, 50 U/mL of rG-CSF, and 2 U/mL of rEP. Cell aliquots were also plated into serum-free medium with 100 U/mL of rIL-3, 100 U/mL of rGM-CSF, 100 U/mL of rG-CSF, 100 U/mL of rM-CSF, and 2 U/mL of rEP. After 14 days of culture, the number of the colonies in each lineage was counted. Each value is the mean \pm SD of six experiments and is expressed as a percentage of the value obtained in serum-containing cultures that gave rise to 51 \pm 4 colonies/100 AB FH cells and in serum-free cultures that gave rise to 44 \pm 6 colonies/100 AB FH cells.

Therefore, in this report, we did not separate CFU-Eo from CFU-Ba and counted the colonies as CFU-Eo/Ba colonies.

Growth characteristics of CFU-M. The serum-containing medium supported CFU-M colonies without the addition of rM-CSF, while the addition of rM-CSF was necessary for the serum-free medium to obtain full CFU-M colony expression (Table 3), and without rM-CSF very few CFU-M colonies were expressed. To address this discrepancy and to investigate whether rM-CSF alone can promote CFU-M colonies in serum-free medium, dose-response experiments with rM-CSF were performed in both serum-containing and serum-free medium.

As shown in Fig 3, in serum-free culture, an increase in the number of CFU-M colonies was present after the addition of 1 U/mL of rM-CSF and a plateau was evident at 100 U/mL. The colonies promoted by the addition of rM-CSF predominantly consisted of CFU-M colonies as shown in Fig 1B. No colonies were present without the addition of rM-CSF, while 67% of the maximum number of CFU-M colonies were present without the addition of rM-CSF in serum-containing medium (Fig 3).

The relation between the number of CFU-M colonies and the number of purified marrow progenitor cells (AB FH cells) plated in serum-free medium in a concentration range of 50 to 800 cells/mL was a straight line through the origin, suggesting a clonal basis for CFU-M development (Fig 4).

**Table 3. Lineage of Purified Progenitor Cells**

<table>
<thead>
<tr>
<th>Progenitor</th>
<th>Serum-Containing Medium</th>
<th>Serum-Free Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroid-mix</td>
<td>1.0 \pm 0.8</td>
<td>1.4 \pm 0.7</td>
</tr>
<tr>
<td>BFU-E</td>
<td>26.9 \pm 4.3</td>
<td>22.9 \pm 9.5</td>
</tr>
<tr>
<td>Small erythroid</td>
<td>16.2 \pm 6.9</td>
<td>11.1 \pm 0.9</td>
</tr>
<tr>
<td>Nonerythroid-mix</td>
<td>3.2 \pm 3.0</td>
<td>8.4 \pm 2.3</td>
</tr>
<tr>
<td>CFU-G</td>
<td>9.1 \pm 4.3</td>
<td>13.9 \pm 3.4</td>
</tr>
<tr>
<td>CFU-M</td>
<td>29.1 \pm 7.3</td>
<td>25.0 \pm 0.9</td>
</tr>
<tr>
<td>CFU-Eo/Ba</td>
<td>12.1 \pm 4.7</td>
<td>16.4 \pm 1.6</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.4 \pm 0.7</td>
<td>1.0 \pm 0.6</td>
</tr>
</tbody>
</table>

The purified cells (AB FH) were cultured in triplicate in 0.5 mL of medium at a concentration of 200 cells/mL in the presence of 50 U/mL of rIL-3, 50 U/mL of rGM-CSF, 50 U/mL of rG-CSF, and 2 U/mL of rEP. Cell aliquots were also plated into serum-free medium with 100 U/mL of rIL-3, 100 U/mL of rGM-CSF, 100 U/mL of rG-CSF, 100 U/mL of rM-CSF, and 2 U/mL of rEP. After 14 days of culture, the number of colonies in each lineage was counted. Each value is the mean \pm SD of six experiments and is expressed as a percentage of the value obtained in serum-containing cultures that gave rise to 51 \pm 4 colonies/100 AB FH cells and in serum-free cultures that gave rise to 44 \pm 6 colonies/100 AB FH cells.
Limiting dilution analysis of CFU-M. The clonal basis of CFU-M growth was further investigated using limiting dilution analysis.\(^9\) The purified AB-FH cells were plated at a variety of concentrations in serum-free medium as indicated in Fig 5, and the percentage of non-responder wells was plotted against the number of cells per well. Straight lines through the origin for CFU-M colonies and clusters were manifested, indicating that rM-CSF acts directly on CFU-M for clonal development.\(^9\)

**DISCUSSION**

The purification method reported here has been characterized by the use of (1) negative panning with concanavalin A-coated plates; (2) positive selection of CD34\(^+\) cells with immunomagnetic microspheres coated with goat antimouse IgG and the release of microspheres from CD34\(^+\) cells by chymopapain treatment; and (3) negative selection of progenitor cells by selected MoAbs. Those characteristics allowed benchtop separation without the need for a cell sorter.

Negative panning with concanavalin A-coated plates was devised to enrich progenitor cells without antibodies before CD34-positive selection. While the purification factor by Con A-negative panning was relatively low, at 1.6-fold, this had an excellent yield at 99\% ± 22\%. We do not know the exact mechanism of enrichment by Con A-negative panning, but based on a trial of other similar reagents, such as phytohemagglutinin, soybean agglutinin, pockweed mitogen, and a combination of these, concanavalin A alone was selected because it provided the highest purification factor and yield.

The introduction of microspheres and the use of chymo-
papain to release microspheres from CD34+ cells is based on the report by Civin et al.20 Chymopapain is known to break the hinge region of Ig.21 When a single incubation was used and varying times and concentrations were tested for removing CD34+ cells, the results varied widely from experiment to experiment (data not shown). The main reason for this instability appeared to be a narrow difference between the pharmacologic capacity of chymopapain to free CD34+ cells and to lyse them. Therefore, we devised a fractionation method with repeated short incubations, and this made it possible to stabilize the recovery and viability of the progenitor cells.

Because the MoAbs used for negative panning were selected empirically, there may be other efficient combinations, but when we tried anti-My31 (CD56) and anti-IL-2 receptor (CD25) MoAb, with the combination listed in Table 1, no increment of progenitor cell purity was obtained. The addition of anti-OKT4 (CD4), anti-My1 (CD15), and anti-My11 (CD45R) reduced the purity of the progenitor cells.

The purified marrow cells contained most of the known progenitor cells, such as CFU-M, CFU-G, CFU-Eo/Ba, BFU-E, small erythroid, erythroid-mix, and nonerythroid-mix (Table 3). CFU-megakaryocyte (CFU-Meg) could be detected and represented up to 3% of the progenitor cells in serum-containing medium, using IIb/IIIa MoAb staining22 (data not shown). Serum-free medium supported 87% ± 11% colony growth, with the same lineage proportion as serum-containing medium (Table 3). There was no significant difference in plating efficiency between serum-free and serum-containing medium, but serum-free medium failed to support CFU-Meg colony expression by purified cells.

CFU-M colonies can be induced from murine marrow cells by human M-CSF.45 In contrast, it has been difficult to show striking evidence of CFU-M stimulation by M-CSF in human bone marrow.44 The expression of CFU-M colonies by human bone marrow cells indicated the presence of progenitors for CFU-M colonies,5,9,10 but the effect of M-CSF on CFU-M colony formation was within the minimum range.43 It has been reported that myeloid cells or monocytes in the marrow inhibit CFU-M colony formation,22 and that M-CSF is present in serum.6 To investigate whether M-CSF stimulates CSF-M colony expression, we combined two methods, using highly purified marrow progenitor cells and a serum-free culture system.

As shown in Fig 3, the presence of serum promoted CFU-M colonies by highly purified marrow cells without the addition of rM-CSF, and with little additional stimulation of CFU-M colonies by the addition of rM-CSF. However, in serum-free medium, the effect of rM-CSF on human marrow progenitor cells was apparent. The positive correlation between the number of CFU-M colonies and the number of purified marrow progenitors indicated a clonal basis for CFU-M development (Fig 4) and this was further investigated using limiting dilution analysis (Fig 5). Even though our marrow progenitor cells were purified to 45% to 67% of the cell population, the effect of added rM-CSF could still occur through an indirect stimulation of the remaining contaminant cells. Therefore, we applied the technique of limiting dilution analysis to identify the presence of an accessory cell mechanism. When the number of purified cells with rM-CSF was plotted against the logarithm of the percentage of CFU-M colony-negative and/or CFU-M cluster-negative wells, a straight line through the origin was apparent, indicating that clonal growth was unaffected by the presence of additional cells and only dependent on the CFU-M itself. The present limiting dilution studies, which show a single hit curve, indicate that a single cell population is involved and that M-CSF acts directly on the CFU-M.

The colony size of CFU-M that we observed in serum-free medium was smaller than that reported by other investigators.43 One possibility is that our studies might be limited to an effect of M-CSF on the more mature progenitors rather than the CFU-M. But when the lineage of the purified marrow progenitors was examined by the addition of rIL-3, rGM-CSF, rG-CSF, rM-CSF, and rEP, the serum-free medium expressed the same percentage of CFU-M colonies compared with those observed in serum-containing medium (Table 3). For this reason, we suspect that some nutritional defect is present in our serum-free medium, not for CFU-M colony expression, but for macrophage proliferation.

In conclusion, we have established a method for purifying human marrow progenitor cells, and using the combination of a serum-free medium, highly purified human marrow progenitors, plus the technique of limiting dilution analysis, we have found that M-CSF acts directly on CFU-M. The purification method presented here is relatively easy to apply in any laboratory with limited expense and laboratory time. With the use of serum-free medium, more precise investigations on the effect of growth factors on progenitor cells can be undertaken than with unpurified marrow cells. It is now also possible to study the pathophysiology of highly purified progenitor cells in a variety of clinical anemias.

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Purification of human marrow progenitor cells and demonstration of the direct action of macrophage colony-stimulating factor on colony-forming unit-macrophage

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