Enrichment of Pluripotent Hemopoietic Progenitor Cells From Human Bone Marrow

By Michael P. Bodger, Ian M. Hann, Roger F. Maclean, and Michael E.J. Beard

Pluripotent hemopoietic progenitor cells (CFU-GEMM, cells forming mixed hemopoietic colonies in methycellulose) from human bone marrow were enriched 90-fold by positive selection on the fluorescence-activated cell sorter using monoclonal antibody RFB-1. Bone marrow cells were separated by cell size, using log 90° light scatter, and the cell fraction containing CFU-GEMM was further separated by relative fluorescence intensity for the RFB-1 antigen. Further enrichment, up to 150-fold, was achieved by depleting bone marrow of T cells and mature myeloid cells prior to RFB-1 selection. These procedures yield a cell fraction containing 51% blast cells, 2% promyelocytes, and 47% undifferentiated (lymphocyte-like) mononuclear cells, although only 1% of the cells formed a mixed colony. CFU-GEMM are strongly positive for the RFB-1 antigen, whereas morphologically identifiable erythroblasts, myeloblasts, and promyelocytes are weakly RFB-1+. This suggests that the relative concentration of the RFB-1 antigen on bone marrow cells is inversely related to their maturity. The greatly increased recovery of CFU-GEMM after the separation of bone marrow by log 90° light scatter and the removal of T cells and mature myeloid cells suggested that accessory cells that normally regulate the cloning efficiency of CFU-GEMM were removed.

THE EXISTENCE of pluripotential hemopoietic stem cells was first demonstrated in the mouse by the in vivo spleen colony-forming (CFU-S) assay. Studies of human myeloid and erythroid differentiation have been promoted by the use of in vitro cloning techniques for committed progenitor cells forming granulocyte-monocytic colonies (CFU-GM) and erythroid colonies (BFU-E and CFU-E), but it is only recently that a cloning technique has been established to enumerate human pluripotential hemopoietic progenitor cells. These cells, termed CFU-GEMM, give rise in culture to mixed hemopoietic colonies containing granulocytic, erythroid, macrophage-monocytic, and megakaryocytic cells when induced by regulatory (colony-stimulating) factors present in leukocyte conditioned medium. CFU-GEMM are capable of limited self-renewal and therefore have features in common with CFU-S.

Studies on the mode of action of regulatory factors would be greatly enhanced by access to purified populations of progenitor cells. Several cell separation methods have been used to isolate hemopoietic progenitor cells. These include physical methods, such as density gradient centrifugation, sedimentation velocity at unit gravity, and density gradient electrophoresis, and immunologic methods, such as binding of antibodies and lectins. The enrichment of CFU-S from rat and murine hemopoietic tissue using immunologic techniques has been recently described, but similar work on humans has been restricted to the isolation of cell fractions containing CFU-GM and BFU-E. We have recently described a monoclonal antibody, RFB-1, which reacts with hemopoietic progenitor cells, including CFU-GEMM. In this article, we describe experiments using the fluorescence-activated cell sorter (FACS) and RFB-1 to enrich CFU-GEMM from human bone marrow.

MATERIALS AND METHODS

Bone Marrow Cells

Bone marrow from informed and consenting adult volunteers (5 to 10 mL) was aspirated from the posterior iliac crest into acid citrate dextrose (1:10, vol/vol). The bone marrow was diluted with 1 vol of phosphate-buffered saline (PBS), and leukocytes were separated by centrifugation (1,000 g, 20 minutes) on 60% Percoll gradients. The interface cells were washed twice in RPMI medium and finally resuspended in RPMI containing 10% fetal calf serum (FCS) at a concentration of 2 × 10^7 cells per mL.

Monoclonal Antibodies

The mouse anti-human monoclonal antibodies used to label subpopulations of bone marrow cells were: (1) RFB-1, an IgG1 class antibody that labels pluripotential and unipotent hemopoietic progenitor cells, myeloblasts, and some promyelocytes; (2) MBG-6, an IgM class antibody that fixes complement and reacts with an antigen on T cells; and (3) CMRF-7, an IgM class antibody that was obtained from a fusion of P3-NS-1/1Ag4.1 myeloma cells and spleen cells from a BALB/c mouse immunized with human leukocytes. The antigen recognized by CMRF-7 is expressed on differentiated granulocytic cells. These include promyelocytes, myelocytes, neutrophils, and some eosinophils, but not basophils or monocytes. Depletion of CMRF-7+ cells from bone marrow in the presence of complement does not inhibit the growth of hemopoietic progenitor cells.
Enrichment of Human CFU-GEMM

Antibody and Complement Treatment of Bone Marrow Cells

Routinely, $50 \times 10^6$ bone marrow cells in 2.5 mL of RPMI medium were incubated with MBG-6 (1:1,000 dilution of ascitic fluid) and CMRF-7 (1:40 dilution of ascitic fluid) for 30 minutes at 4°C (the optimal dilution for each antibody was determined from titration curves using antibody-treated bone marrow cells as targets in a complement-mediated cytolytic assay). The cells were washed once in 5% FCS/RPMI, resuspended in 2.5 mL FCS/RPMI, and incubated at 37°C for 45 minutes, after which the addition of an equal volume of rabbit serum (Buxted Rabbit Co., Buxted, Sussex, UK) as a source of complement. After a final wash in FCS/RPMI, the cells were incubated with RFB-1 for analysis and separation on the FACS (see below).

Cell Sorting of Bone Marrow

Bone marrow cells were separated according to relative low-angle light scatter or log 90° light scatter and relative fluorescence intensity for RFB-1 antigen on a FACS IV (Becton-Dickinson, FACS System, Sunnyvale, Calif). Briefly, bone marrow cells ($50 \times 10^6$ cells in 10% FCS/RPMI) were incubated with RFB-1 (1:250 dilution of ascites) for 15 minutes at 4°C. The cells were washed twice in ice-cold medium and stained with goat anti-mouse Ig conjugated to fluorescein isothiocyanate (G anti-M Ig-FITC; Nordic Immunology, Tilburg, The Netherlands). The cells were finally washed twice in ice-cold medium, filtered through 95µm Simonester mesh (Henry Simon Ltd, Stockport, Cheshire, England), and resuspended at $4 \times 10^6$ cells per mL. Control marrow samples were stained with 1:250 dilution of ascites from a non-antibody-producing hybrid clone and stained with second layer G anti-M Ig-FITC. The cells were maintained at 4°C to prevent capping of the antibody. Cells were sorted on the FACS according to low-angle (forward) light scatter or log 90° light scatter and relative fluorescence intensity for RFB-1 antigen on a FACS IV (Becton-Dickinson, FACS System, Sunnyvale, Calif).

CFU-GEMM Assay

Bone marrow pluripotential progenitor cells were assessed using a mixed colony assay as previously described. Bone marrow cells were incubated in IMDM containing 0.9% methylcellulose as viscous support, 10% FCS, 20% fresh human plasma, and 5% conditioned medium from phytohemagglutinin-stimulated leukocytes. Cultures were set up with $10^4$ to $2 \times 10^6$ cells in 1-mL vol in 35-mm Petri dishes (Nunc, Roskilde, Denmark), and erythropoietin (1 U/mL; Connaught Laboratories Ltd., Willowdale, Ontario, Canada) was added at the start of the culture. CFU-GEMM-derived colonies containing mixed erythroid, granulocytic-monocytic, and megakaryocytic cells were identified by their morphology and on stained smears (see below) and scored at day 14. Colonies derived from CFU-GM, BFU-E, and CFU-Eos were also assessed in the mixed assay. The assay is linear, with extrapolation through the origin.

Cell Staining

Smears of bone marrow samples prior to and after cell sorting were prepared using a cytocentrifuge (Shandon Southern Products Ltd., Cheshire, England). Cell colonies from the CFU-GEMM assay were removed from the culture using a micropipette, transferred onto glass slides, and gently air-blown to spread the cells. The smears were stained with May-Grünwald-Giemsa, and cell types were identified under 100× magnification. Bone marrow differential cell counts were performed on 200 cells/smear.

RESULTS

Light Scatter Analysis of Bone Marrow Cells

Samples of bone marrow were initially analyzed and sorted on the FACS according to low-angle (forward) and log 90° light scatter (measurements of cell size). Marrow cells were resolved into two peaks according to low-angle light scatter (Fig 1A). Peak A contained erythrocytes and peak B contained all the nucleated cells. When analyzed by log 90° light scatter, the nucleated cells were further resolved into two peaks (Fig 1B). Peak I contained mainly lymphocyte-like cells and erythroblasts, and peak II contained a mixture of immature and mature myeloid cells (Table 1). Approximately 95% of CFU-GEMM were observed in the right-hand side of peak I ($I_a$), and this resulted in a mean 13-fold enrichment (range, ten to 17) and a recovery five times the expected value. This fraction also contained 50% of the BFU-E and CFU-GM.

RFB-1 Expression on Progenitor Cells

In the next series of experiments, bone marrow cells were stained by indirect immunofluorescence with RFB-1, and the fluorescence profile and morphology of cells in fraction $I_a$ were analyzed. Three populations of cells were observed (Fig 1C). Approximately 90% of the cells in fraction 1 were mature myeloid cells, and no colony-forming cells were seen (Table 2). Cells in fraction 2 consisted of lymphocyte-like cells, erythro-
blasts, and immature myeloid cells, but only a small number of BFU-E and CFU-GM were observed. Fraction 3 contained all the CFU-GEMM, as well as the majority of CFU-GM, BFU-E, and CFU-Eos. This resulted in a mean 66-fold enrichment (range 51 to 105) of CFU-GEMM compared with unfractonated marrow and a recovery 3.3 times the expected value. FACS analysis showed that CFU-GEMM were enriched in a strongly RFB-1+ “small-sized” cell fraction (Fig 2). Morphologically, 55% of the cells in fraction 3 were lymphocyte-like cells, and the remainder were immature myeloid and erythroid cells. Further-
ther separation of the cells in fraction 3 showed that 70% of CFU-GEMM could be recovered in the right-hand side (fraction $3_r$), with a mean 88-fold enrichment (range 66 to 130). This fraction contained 60% lymphocyte-like cells, undifferentiated blast cells, and immature myeloid cells. Approximately 20% of the lymphocyte-like cells expressed the MBG-6 (T cell) antigen.

**Removal of T Cells and Myeloid Cells**

The effect of removing T cells and mature myeloid cells prior to RFB-1 selection of CFU-GEMM was studied using cytolytic monoclonal antibodies MBG-6 and CMRF-7. Virtually all cells in peak II of the log $90^\circ$ light scatter profile of bone marrow cells were removed (Fig 3A). More than 90% of the cells in peak II and 10% of the cells in peak I were promyelocytes, myelocytes, and metamyelocytes, and their removal after antibody treatment was morphologically verified (Table 3, row 2). Treatment of bone marrow cells with

Table 3. Cell Morphology and Distribution of CFU-GEMM in Fraction $I_4$ Following the Removal of MBG-6* and CMRF-7* Cells

<table>
<thead>
<tr>
<th>Unseparated</th>
<th>Ab Treatment</th>
<th>$I_4$ Subtraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated blast cells</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Polymorphs</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monocytes</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Erythroblasts</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>31</td>
<td>42</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Percent cells</td>
<td>100</td>
<td>43</td>
</tr>
</tbody>
</table>

CFU-GEMM per $10^6$ cells

<table>
<thead>
<tr>
<th>Enrichment</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5 x 2</td>
<td>100</td>
</tr>
<tr>
<td>57 ± 12</td>
<td>14</td>
</tr>
<tr>
<td>30 ± 7</td>
<td>5.5 x 148</td>
</tr>
<tr>
<td>816 ± 143</td>
<td>430</td>
</tr>
<tr>
<td>ND</td>
<td>222</td>
</tr>
</tbody>
</table>

Differential counts and colony results from four experiments. The unseparated cells were labeled with RFB-1 and G anti-M Ig-FITC. Ab treatment: antibody treatment; the marrow was depleted of MBG-6* and CMRF-7* cells and labeled with RFB-1 and G anti-M Ig-FITC. Neither the unseparated or antibody-treated cells were passed through the FACS. Enrichment and yield calculations as in footnote to Table 1.

ND, not determined.

MBG-6 and CMRF-7 yielded a tenfold enrichment of CFU-GEMM.

Following the removal of T cells and myeloid cells from the marrow samples, the cells were stained with RFB-1, and the fluorescence profile and the morphology of the cells in fraction $I_8$ were examined. One population of strongly RFB-1* cells was observed (Fig 3B) and corresponded to fraction 3 of Fig 1C. The right-hand side of this peak contained more than 95% of CFU-GEMM and resulted in a mean 150-fold enrichment (range, 134 to 168). This fraction contained approximately 50% blast cells and 50% lymphocyte-like cells. No MBG-6* T cells were observed.

**DISCUSSION**

In these experiments, we have shown that pluripotential hemopoietic progenitor cells from human bone marrow can be enriched up to 150-fold on the FACS using a combination of light scatter and relative fluorescence intensity for the RFB-1 antigen. Bone marrow cells were initially separated on the FACS according to log $90^\circ$ light scatter. Morphological examination of the cells showed that log $90^\circ$ light scatter was a useful measurement of cell size, although the intensity of light scattered at $90^\circ$ is also dependent on cell refractive index and the number of internal reflective surfaces. CFU-GEMM were predominantly isolated in a cell fraction that was strongly RFB-1*.
finding and the earlier observation that the expression of RFB-1 antigen on morphologically identifiable myeloid cells decreases as the cells become more mature\textsuperscript{18} suggests that the concentration of RFB-1 antigen on bone marrow cells is inversely related to their relative state of maturity.

Selection of strongly RFB-1\textsuperscript{+} cells in fraction I\textsubscript{R} (single step procedure) resulted in an 88-fold enrichment of CFU-GEMM and a cell suspension containing mainly lymphocyte-like cells, blast cells, and promyelocytes. Selection of strongly RFB-1\textsuperscript{+} cells after treatment with MBG-6 and CMRF-7 antibodies (two-step procedure) resulted in a 150-fold enrichment of CFU-GEMM and a cell suspension containing blast cells and lymphocyte-like cells only. The expression of MBG-6 on a small percentage of cells following the single-step procedure indicated that T cells were present in the cell suspension. However, no T cells were observed in the cell suspension following the two-step selection of CFU-GEMM. In addition, RFB-1 is unreactive with pre-B and B cells.\textsuperscript{18} Therefore, the majority of cells with lymphocyte-like morphology are probably undifferentiated mononuclear cells, although the presence of immature T cells and lymphocyte progenitor cells cannot be excluded. This suggestion is supported by morphological analysis of pluripotential stem cell candidates in the rat\textsuperscript{13} and mouse\textsuperscript{24} and CFU-GM/BUF-E enriched fractions from mouse bone marrow.\textsuperscript{25}

Several groups have used positive and negative selection techniques to enrich for CFU-GM, BFU-E, and CFU-E from human hemopoietic tissue.\textsuperscript{15-17,26} These studies showed that the majority of myeloid colony- and cluster-forming cells are larger than lymphocytes and consist primarily of undifferentiated blast cells and promyelocytes. This has also been clearly shown in FACS-separated rat bone marrow, where CFU-GM reside in the "large" cell fraction, comprising myeloblasts and promyelocytes and separate from stem cells that reside in the undifferentiated mononuclear cell population.\textsuperscript{13} In our experiments, the mixed colony assay also supported the growth of CFU-GM, BUFE, and BFU-Eos. Whereas greater than 95\% of CFU-GEMM resided in fraction I\textsubscript{R} of the log 90\degree light scatter profile (Fig 1B), CFU-GM, BFU-E, and CFU-Eos were distributed in all four fractions. This suggests that these progenitor cells are more heterogeneous with respect to size than CFU-GEMM.

Despite a 150-fold enrichment of CFU-GEMM in the "purest" fraction, only one cell in 100 formed a mixed colony; therefore, the cloning efficiency for CFU-GEMM remains very low. Cloning efficiencies refer specifically to the efficiency with which cells of a known uniform type are able to grow in vitro and therefore should only be applied to homogeneous cell populations.\textsuperscript{27} The present cell fraction is not homogeneous, and therefore, the true cloning efficiency for CFU-GEMM could be higher, albeit low. Although CFU-S from murine and rat bone marrow have been greatly enriched on the FACS, the cloning efficiency in the in vivo spleen colony assay also remains very low.\textsuperscript{13,14} Therefore, the CFU-S fractions are by no means pure. This could be due to the low starting frequency of CFU-S and CFU-GEMM and the inefficiency of the respective stem cell assays, certainly the mixed colony assay. In contrast, cloning efficiencies of 0.1 to 0.47 for human and murine cell fractions enriched for unipotent progenitor cells have been achieved.\textsuperscript{15,17,25,26} However, considerably higher starting frequencies of progenitor cells were observed.

The greatly increased recovery of CFU-GEMM in fraction I\textsubscript{R} compared with other progenitor cells was surprising (Table 1). Whereas a threefold to fourfold enrichment of CFU-GEMM was expected, a 13-fold enrichment was observed. One explanation is that accessory cells that normally regulate the cloning efficiency of CFU-GEMM were removed. That this was likely was shown after treating bone marrow samples with anti-T cell and anti-myeloid cell monoclonal antibodies prior to selection by cell scatter. A tenfold enrichment of CFU-GEMM was achieved following the removal of T cells and mature myeloid cells. This confirms earlier observations showing that monocyte depletion and the removal of E rosette-positive cells from bone marrow prior to plating increased the number of mixed colonies in vitro.\textsuperscript{29} Observed enrichment exceeding predicted enrichment of CFU-S has also been reported in the rat, and it was suggested that FACS sorting of Thy-1\textsuperscript{+} bone marrow cells removed a weakly Thy-1\textsuperscript{+} suppressor cell population but not a strongly Thy-1\textsuperscript{+} amplifier cell population.\textsuperscript{13} In addition, there is evidence that two functionally distinct subpopulations of T cells have enhancing and suppressive effects on human BFU-E.\textsuperscript{30}

In summary, we have achieved considerable enrichment of human pluripotent hemopoietic progenitor cells using positive selection with the RFB-1 monoclonal antibody. The procedure can provide a large number of immature bone marrow cells. These could be used to raise further monoclonal antibodies to human stem cell-related antigens and allow more detailed studies on the antigenic phenotype and biochemical characteristics of pluripotential hemopoietic stem cells and the effect of purified colony-stimulating factors on stem cell differentiation.

ACKNOWLEDGMENT
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