This study reports the development of an assay, the Pre-colony-forming unit (CFU) assay, which detects human hematopoietic precursors. The Pre-CFU assay is based on the observation that precursors to CFU-granulocyte-macrophage (CFU-GM) that are undetectable in clonogenic assays differentiate into CFU-GM preferentially following treatment in suspension culture with recombinant human interleukin-1α (rhIL-1α) combined with rhIL-3. Using the Pre-CFU assay, hematopoietic precursors were detected in human bone marrow depleted of CFU-GM progenitors and differentiated hematopoietic elements via 4-hydroperoxycyclophosphamide treatment coupled with selection for CD34+ cells (4-HCm/CD34+ marrow). Additionally, the Pre-CFU assay detected recovery of hematopoiesis substantially earlier than the CFU-GM assay in primates following myeloablation with 5-flourouracil. The Pre-CFU assay was used to assess purification of a phenotypically defined hematopoietic precursor population, the lin-CD34+ population. The lin-CD34+ population lacks detectable surface markers for T-cell, B-cell, natural killer cell, and myeloid lineage, possesses the CD34 antigen, is devoid of CFU-GM progenitors, and yields Pre-CFU assay values comparable with 4-HCm/CD34+ marrow. Using a combination of phenotypic analysis and Pre-CFU assay analysis, the action of rhIL-1α plus rhIL-3 treatment on lin-CD34+ cells was further characterized. The data indicate that rhIL-1α plus rhIL-3 treatment induces proliferation and differentiation of early hematopoietic precursors into progenitors and terminally differentiated cells, without inducing a significant expansion of the precursor population itself.

**A NUMBER OF EXPERIMENTAL systems indicate that hematopoiesis occurs via proliferation and differentiation of a small number of pluripotent stem cells into progressively restricted progenitor populations and, ultimately, into terminally differentiated cells.** In the murine system, a number of in vivo and in vitro assays have been used to define the stem cell and progenitor cell developmental compartments. The pluripotent stem cell population has been defined by its ability to reconstitute hematopoiesis in competitive reconstitution assays and in serial bone marrow transplant (BMT) recipients. The D12 colony-forming unit-spleen (CFU-S) and D8 CFU-S assays, meanwhile, appear to detect more committed hematopoietic precursors. In vitro assays that appear to assess murine reconstituting stem cells and early precursors include the long-term BM culture initiation assay and the high proliferative potential colony (HPPC) assay. The CFU-A assay, the CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) assay, and the CFU granulocyte-macrophage (CFU-GM) assay appear in general to detect progressively restricted progenitor cell populations.

In the human setting, the delineation of stem cell, precursor cell, and progenitor cell populations has remained limited. Identifying and quantitating the human pluripotent stem cell population has not been possible to date because in vivo BMT reconstitution studies with limiting dilutions of purified cells or competitive reconstitution assays cannot be performed. The traditionally used CFU-GEMM and CFU-GM clonogenic assays have not consistently predicted the behavior of reconstituting stem cells in allogeneic and purged autologous BMT recipients. As in the murine system, these assays appear to assess developmentally mature progenitor populations. Several assays including the human blast cell assay, the human HPPC assay, and the xenogeneic mouse BMT model have been developed for assessment of precursors to the CFU progenitor compartment. All these assays to date remain poorly characterized, and the relationship of cells detected in these assays to pluripotent stem cells remains unclear.

Recently, in an effort to improve hematopoietic gene transfer and BMT protocols, we have initiated a program aimed at identifying and expanding in vitro, primitive human hematopoietic cells. As a prerequisite to these studies, we have developed a simple assay for the detection of early hematopoietic precursors. This assay, termed the Pre-CFU assay, is based on prior observations in the murine system identifying a hierarchical response of precursor and progenitor cell populations to soluble cytokines. Early hematopoietic precursors proliferate and differentiate in response to interleukin-1 (IL-1) or IL-6 in synergy with IL-3 while progressively committed populations respond preferentially to granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF), and macrophage CSF (M-CSF). In this report, we demonstrate that human and primate hematopoietic precursors undetectable in CFU-GM assays differentiate into CFU-GM preferentially in response to recombinant human IL-1α (rhIL-1α) combined with rhIL-3 treatment. The generation of nascent CFU-GM serves as an index of precursors to CFU-GM. Using the Pre-CFU assay, we have purified a phenotypically defined human hematopoietic...
prerogatory population and initiated functional characterization of these cells.

**MATERIALS AND METHODS**

**Secondary CFU Assay**

**BM cells.** Human BM cells were obtained from consenting normal donors of BM for allogeneic BMT and normal volunteers. Samples were collected under general or local anesthesia from the iliac crest into heparinized saline (1 mL/5 mL BM). Primate BM was obtained from femurs and iliac crests of Cynomolgus primates under ketamine anesthesia (typically 10 mg/kg) into heparinized saline as above.

4-HC<sup>-</sup>/CD34<sup>+</sup> cell preparation. BM cells (1 x 10<sup>6</sup>) obtained as above were washed twice in phosphate-buffered saline (PBS) and resuspended in Iscove’s modified Dulbecco’s medium (IMDM) at 2 x 10<sup>6</sup> cells/mL. Freshly reconstituted 4-hydroxycyclophosphamide (4-HC) was added to a concentration of 100 µmol/L. Cells were treated at 37°C for 30 minutes with gentle mixing. Treated cells were then layered on Ficoll-Hypaque (Hyclone, Logan, UT) and centrifuged for 25 minutes at 1,500 rpm. CD34<sup>+</sup> cells were then isolated by washing the light density 4-HC-treated cells twice with PBS and resuspending in PBS/2% fetal calf serum (FCS) with a 1:500 dilution of ICH3 (R. Levinsky, London).<sup>18</sup> Cells were incubated 30 minutes at 4°C then layered on 100-mm tissue culture-treated dishes previously coated at 4°C overnight with goat antihorse IgG (Boehringer Mannheim, Indianapolis, IN) 100 µg/mL. CD34<sup>+</sup> cells were allowed to adhere for 4°C for 1 hour. Nonadherent CD34<sup>+</sup> cells were then removed with gentle washing with PBS four times via a 10-mL pipette and adherent cells recovered with vigorous washing six times via a 25-gauge needle.

**Secondary CFU assays and the Pre-CFU assay.** Precursor-enriched BM cells (1 x 10<sup>6</sup>) (human CD34<sup>+</sup>/4-HC<sup>-</sup>) cells, lin<sup>-</sup>CD34<sup>+</sup> cells, or primo post-furocaracil (5-FU) cells were plated for CFU-GM with 1.000 U/mL rhGM-CSF (Amgen, Thousand Oaks, CA) in 0.36% agarose in triplicate. Colonies (scored as >50 cells) at 14 days of incubation were counted as the total colony frequency. Simultaneous to plating D<sub>0</sub> CFU colonies, 1 x 10<sup>6</sup> cells were placed in suspension culture in IMDM/10%FCS with cytokines at doses listed below. For the Pre-CFU Assay, rhIL-1β (100 U/mL; Syntex, Palo Alto, CA) + rhIL-3 (50 ng/mL; Genetics Institute, Cambridge, MA) were used. At day 7 of suspension growth, cells were counted and 1 x 10<sup>6</sup> cells from each suspension culture were plated for CFU-GM as described above. The colony number per total suspension culture per original 10<sup>6</sup> cells was recorded as the secondary CFU number. Cytokines used in suspension cultures in comparison with IL-1 + IL-3 included: GM-CSF, 1,000 U/mL (Amgen); G-CSF, 1,000 U/mL (Amgen); CSF-1, 1,000 U/mL (Amgen); and IL-6 5 U/mL (Immunex, Seattle, WA). Maximal IL-6 activity was determined by combining IL-6 plus IL-3 (50 ng/mL) over a range of 1 U IL-6/mL to 10,000 U IL-6/mL at log intervals in secondary CFU assays as described above. IL-6 activity was confirmed using the B9 cell proliferation assay.<sup>19</sup> The Pre-CFU assay value is the IL-1 + IL-3 secondary CFU number minus D<sub>0</sub> CFU number.

**Primate Studies**

**Primates were cared for as described previously in accordance with Nation Institutes of Health (NIH) and Institute guidelines.**<sup>20,21</sup> Young male Cynomolgus primates were treated with 5-FU 75 mg/kg/d for 2 days via single intravenous (IV) bolus daily. CFU-GM, IL-1 + IL-3 secondary CFU, and IL-3 secondary CFU values were determined as described above before 5-FU treatment and at weekly intervals thereafter. Primates receiving IL-3 therapy post-5-FU were treated with IL-3 (20 µg/kg IV bolus daily) for 14 days as described elsewhere.<sup>21</sup>

**Human lin<sup>-</sup>CD34<sup>+</sup> Population Purification**

**Soybean agglutination.** Unfractionated BM cells were suspended in Hanks’ Balanced Salt Solution (HBSS) at a concentration of 200 x 10<sup>6</sup>/mL, mixed with 2 µg/mL solution of soybean agglutinin (Vector Laboratories, Burlington, CA), and gently swirled 1 to 3 minutes until visible agglutination occurred.<sup>22</sup> The suspension was then layered on top of 5% bovine serum albumin (BSA; Sigma, St. Louis, MO)/HBSS and the agglutinated cells allowed to settle by gravity. The unagglutinated cells (soybean agglutinin negative [SBA<sup>-</sup>] cells) at the interface were then collected and suspended in 0.2 mol/L galactose to remove the soybean lectin. The cells were further washed twice in PBS.

**Removal of CD33<sup>+</sup> and M01<sup>+</sup> cells with panning and complement-mediated cytotoxicity.** SBA<sup>-</sup> cells were incubated for 45 minutes at 4°C in a 20 µg/50 x 10<sup>6</sup> cells of the mouse anti-CD33 antibody MY-9 (Becton Dickinson, San Jose, CA) and a 1:500 dilution of M01 (Coulter, Hialeah, FL) in PBS/2% FCS and panned on 100-mm goat anti-lg plates as described above, with collection of nonadherent cells. Nonadherent cells were then incubated for 30 minutes in a 1:6 dilution of rabbit complement (Cedar Lanes, Westbury, NY) at 37°C to remove residual CD33<sup>+</sup>M01<sup>+</sup> cells and then washed three times in PBS.

**CD34<sup>+</sup> panning.** SBA<sup>-</sup>CD33<sup>-</sup>M01<sup>-</sup> cells were panned for CD34<sup>+</sup> with IC/C3 at a 1:500 dilution exactly as described above. Adherent cells were termed lin<sup>-</sup>CD34<sup>+</sup> cells.

**Fluorescence Activated Cell Sorter (FACS) Analysis**

Immunofluorescence staining and FACS analysis to detect residual lineage marker-positive cells in the lin<sup>-</sup>CD34<sup>+</sup> population was performed on a FACScan (Becton Dickinson) apparatus using a 488-nm incident laser with data collection and analysis as described by Parks et al.<sup>23</sup> Direct immunofluorescence staining of lineage-specific markers was used to eliminate background staining from residual antibody remaining on the cells from the panning procedures. Staining was performed at 4°C using 20 µL of antibody in 50 µL of cell suspension in PBS/4% FCS/0.1% azide. Antibodies used include: phycoerythrin-MY9 for myeloid cells, fluorescein isothiocyanate (FITC) anti-CD22 for B cells, FITC anti-Leu2a and FITC anti-Leu3a for T cells, and FITC anti-CD16 for natural killer (NK) cells (all antibodies from Becton Dickinson). Forward and 90° light scatter gates and background fluorescence were determined using unstained lin<sup>-</sup>CD34<sup>+</sup> cells. The positive gate cutoff was set at 99% of the background population. FITC-MY10 (anti-CD34), graciously provided by Peter Landscörp, Vancouver, Canada, was used at 5 µg/10<sup>6</sup> cells.

**Primitive Progenitor Cell Assays**

**HPPC assay.** The human HPPC assay was performed as described.<sup>22</sup> Unfractionated (5 x 10<sup>6</sup>) or 1 x 10<sup>6</sup> lin<sup>-</sup>CD34<sup>+</sup> cells were plated in 60-mm plates in double-layered 3% agarose. IL-3 (50 ng/mL) and GM-CSF (1,000 U/mL) were included in the bottom layer and cells were placed in the top. Plates were incubated 28 days at 37°C in 7%O<sub>2</sub>/5%CO<sub>2</sub> and HPPC were scored as high density colonies greater than 0.5 mm in diameter. All cultures were performed in triplicate.

**Human long-term BM culture assay (LTBMC assay).** Cells (5 x 10<sup>6</sup> and 5 x 10<sup>6</sup>) were placed in duplicate in 1-cm<sup>2</sup> wells with previously established 3- to 5-week-old allogeneic stromal layers.
Stromal layers were started from $2 \times 10^6$ unfractonated BM cells plated in Gartner’s media with weekly media changes at 37°C and depleted of hematopoietic elements by weekly removal of all nonadherent cells after week 2 of culture. Stroma was irradiated at 1,000 rad immediately before seeding with test hematopoietic cells. LTBMCs were then maintained at 37°C with weekly media changes and semi-depopulation. CFU-GM at 4 weeks was determined as described above.

**Statistical Analysis**

Statistical analyses were performed using the Student’s paired t-test.

**RESULTS**

**Secondary CFU Assay of CD34+/4-HCtreated Human BM**

4-HC treatment has been demonstrated previously to deplete the majority of CFU-GM forming progenitors while preserving stem cells capable of hematopoietic reconstitution. In addition, CD34+ selection has been shown to enrich reconstituting stem cells in primate and human studies. We used the combination of 4-HC treatment (100 μmol/L) coupled with positive selection for CD34+ cells to deplete the CFU-GM forming population while preserving early hematopoietic precursors. Human 4-HCtreated/CD34+ selected BM was exposed to a variety of cytokine combinations in suspension phase to determine which would generate the greatest number of CFU-GM in subsequent clonogenic assays. Table 1 demonstrates the response of 4-HCtreated/CD34+ marrow to cytokines alone and in combinations. rhIL-1α at 100 U/mL + rhIL-3 at 50 ng/mL consistently generated the highest number of secondary CFU-GM. rhIL-6 added to 50 ng/mL rhIL-3 generated CFU-GM at a frequency similar to IL-3 alone at doses of IL-6 between 1 and 10,000 U/mL. CFU-GM following IL-1 + IL-3 treatment exhibited typical CFU-GM morphology by Wright-Giemsa staining.

**Correlation of Precursor Populations Identified by the Secondary CFU Assays and the CFU-GM Assay to Hematopoietic Stem Cells**

The Cynomolgus primate has been demonstrated previously to be an authentic model of human hematopoiesis. In vivo 5-FU treatment has been demonstrated in both primate and murine models to eliminate mature hematopoietic cells and CFU-GM level progenitors while preserving reconstituting stem cells. Using the primate model, we investigated the relationship between populations detected by the IL-1 + IL-3 secondary CFU assays, IL-3 secondary CFU assays, and CFU-GM assays to the stem cells surviving in vivo 5-FU treatment. Secondary CFU and CFU-GM assays were performed using primates obtained at serial time points following cytoablative with 5-FU. Table 2a demonstrates that populations with high IL-1 + IL-3 secondary CFU values are detectable at least 7 days following cytoablation, ≥ 14 days before the onset, and peaking of CFU-GM populations. IL-1 + IL-3 secondary CFU values at all time points were greater than IL-3 secondary CFU values ($P < .02$ at day 7 post-5-FU). A similar pattern of IL-1 + IL-3 responsive elements with an accelerated recovery of CFU-GM was observed in primates recovering in vivo IL-3 therapy following 5-FU cytoablation (Fig 1B).

**Purification of a Human Hematopoietic Precursor Population**

Subsequent to demonstrating a population of precursors to CFU-GM functionally defined by its response to the combination of IL-1 + IL-3, a phenotypically defined population was identified and purified that exhibited IL-1 + IL-3 secondary CFU values similar to the 4-HCtreated/CD34+ population. Andrews et al have identified a human Pre-CFU population in LTBMCs defined by the CD34+CD33- phenotype. In addition, numerous murine and human studies have demonstrated stem cell enrichment via removal of cells committed to lymphoid and myeloid lineages. We purified a hematopoietic precursor population, the lin CD34+ population, using a combination of depletion of lineage committed elements (including CD33+ populations), and enrichment for CD34+ cells. Soybean agglutination was used to remove erythroid, mature myeloid, and the majority of the lymphoid populations. Soybean agglutination has previously been demonstrated to remove the majority of mature myeloid and erythroid elements, and more than 90% of T, B, and NK cells. In addition, this procedure has previously been demonstrated to provide full hematopoietic reconstitution with 10-fold fewer cells than required for unfractionated marrow in primate autologous BMT recipients and human allogeneic BMT recipients (unpublished observations). Following soybean agglutination, depletion of the remaining CD33+M01+ myeloid cells were accomplished with one round of panning followed by one round of complement-mediated cytotoxicity. This procedure removed the majority of directly forming CFU-GM elements (Table 2). Enriching for CD34+ cells with positive panning yielded populations

---

Table 1. Generation of Secondary CFU-GM from 4-HCtreated/CD34+ Human BM Following Cytokine Treatment in Suspension Cultures

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>D0 CFU-GM</th>
<th>Secondary CFU-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>1.5 ± 1.5</td>
<td>26.3 ± 11.2</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1.5 ± 1.5</td>
<td>24.4 ± 9.0</td>
</tr>
<tr>
<td>IL-3</td>
<td>1.5 ± 1.5</td>
<td>66.0 ± 16.6</td>
</tr>
<tr>
<td>M-CSF</td>
<td>1.5 ± 1.5</td>
<td>2.8 ± 1.7</td>
</tr>
<tr>
<td>IL-1</td>
<td>1.5 ± 1.5</td>
<td>12.1 ± 4.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.5 ± 1.5</td>
<td>11.4 ± 5</td>
</tr>
<tr>
<td>IL-1 + G-CSF</td>
<td>1.5 ± 1.5</td>
<td>52.5 ± 26.4</td>
</tr>
<tr>
<td>IL-1 + GM-CSF</td>
<td>1.5 ± 1.5</td>
<td>31.0 ± 19.4</td>
</tr>
<tr>
<td>IL-1 + IL-3</td>
<td>1.5 ± 1.5</td>
<td>119.3 ± 52.8</td>
</tr>
<tr>
<td>IL-1 + M-CSF</td>
<td>1.5 ± 1.5</td>
<td>21.9 ± 3.3</td>
</tr>
<tr>
<td>IL-6 + IL-3</td>
<td>1.5 ± 1.5</td>
<td>68.3 ± 25.3</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of three separate experiments. GM-CSF (1,000 U/mL) was used in all CFU-GM assays. IL-1 + IL-3 secondary CFU-GM mean was greater than all other cytokine treatments at $P < .05$ except IL-3 alone ($P < .005$) and IL-1 + G-CSF ($P < .116$). The large $P$ value for IL-1 + G-CSF is due to the large IL-1 + G-CSF standard deviation. In all experiments analyzed to date, IL-1 + IL-3 has consistently generated more secondary CFU than IL-1 + G-CSF.
greater than 90% CD34+ by FACS analysis. The resulting lin−CD34+ population possessed a uniformly small blastic appearance on Wright-Giemsa stained cytospins. Immunofluorescence staining and FACS analysis showed the absence of detectable markers for myeloid (CD33, MO1), T-cell (CD4, CD8), B-cell (CD22), and NK-cell (CD16) lineages. Lin−CD34+ cells demonstrated a low 90° angle light scatter and low to medium forward angle light scatter consistent with prior descriptions of Pre-CFU cells.\textsuperscript{14,26} Typically, 0.1% to 0.2% of the unfractionated population was recovered as compared with the predicted less than 1% lin−CD34+ cells detected by FACS analysis of un fractionated cells.

Table 2. \( D_0 \) CFU-GM and IL-1 + IL-3 Secondary CFU-GM Values for Populations Progressively Enriched for Hematopoietic Stem Cells

| Fraction          | \( D_0 \) CFU-GM | IL-1 + IL-3 Responsive Secondary CFU
|-------------------|------------------|----------------------------------------
| SBA                | 114.6 ± 19       | 308 ± 65.3                             |
| SBA−CD33+MO1−      | 49.6 ± 9.5       | 272 ± 123.4                            |
| lin−CD34+          | 10.7 ± 4.1       | 396.5 ± 96                             |

Values shown are the mean and standard error of four separate experiments. Secondary CFU-GM is following IL-1 + IL-3 prestimulation. \( D_0 \) CFU-GM and secondary CFU-GM readout is with GM-CSF (1,000 U/mL). The difference between \( D_0 \) CFU-GM and secondary CFU-GM is significant at \( P < .05 \) only for the lin−CD34+ population.

Characterization of the Response of lin−CD34+ Cells to IL-1 + IL-3

Isocye et al have recently reported that murine Pre-CFUmix precursors increase in number in response to IL-1 + IL-3 treatment in suspension culture.\textsuperscript{16,29} We investigated whether IL-1 + IL-3 treatment of human lin−CD34+ precursor cells induced mainly differentiation of lin−CD34+
precursors into detectable CFU-GM progenitors or if an actual increase in precursors occurred as well. lin CD34+ cells were treated with IL-1 (100 U/mL) plus IL-3 (50 ng/mL) in serum containing media for 7 days. To define the IL-1 + IL-3 treated population phenotypically, Wright-Giemsa staining and FACS analysis of CD33+ and CD34+ proportion were performed. Table 3 demonstrates that while the total cell number of the lin CD34+ population following IL-1 + IL-3 treatment increases on the average threefold, the majority of cells have differentiated into mature myeloid cells as seen by the high proportion of CD33+ cells, the decreased CD34+ proportion, and the presence of mainly mature myeloid elements on Wright-Giemsa-stained cytopsins of the treated suspension culture. In addition, IL-1 + IL-3 secondary CFU assay analysis of cytokine-treated cells demonstrates a decline in IL-1 + IL-3 responsive hematopoietic elements. IL-1 + IL-3 treatment did not completely exhaust the IL-1 + IL-3 responsive cells, however, as secondary CFU were generated during the IL-1 + IL-3 secondary CFU assay of postcytokine-treated cells.

**DISCUSSION**

The ability to characterize and purify human hematopoietic stem cells is essential for clarifying the biology of the human hematopoietic system and for improving BMT and hematopoietic gene therapy procedures. In the present study, we present a relatively quick (21 days) and technically simple method for assessing early hematopoietic precursors: the Pre-CFU assay (the IL-1 + IL-3 secondary CFU assay). Previous studies of the human hematopoietic system have used the CFU-GM assay, the CFU-GEMM assay, the HPPC assay, the blast cell assay, or the LTBM assay to define the experimental outcome. The CFU-GM and CFU-GEMM assays appear to detect relatively mature progenitor compartments and have not consistently predicted the outcome of BMT.8,10 The long-term culture assay is time consuming, requiring a minimum of 6 to 8 weeks for interpretation, and the complexity of culture conditions and CFU-GEMM assays appear to detect relatively mature progenitor compartments and have not consistently predicted the outcome of BMT.8,10 The long-term culture assay is time consuming, requiring a minimum of 6 to 8 weeks for interpretation, and the complexity of culture conditions often leads to significant intra- and interindividual variations in culture outcome. The blast cell assay is labor intensive and the relationship of human blast colony progenitors to reconstituting stem cells remains unclear. In our studies, the HPPC assay did not distinguish between IL-3-responsive elements and IL-1 + IL-3 responsive elements as did the Pre-CFU assays of 4-HC(terminal)/CD34+ human marrow and primate marrow recovering from 5-FU cytoreduction (see discussion below). Several studies using the murine model indicate that IL-1 + IL-3 responsive populations are developmentally more primitive than IL-3 alone responsive populations.15,20 The human HPPC assay then appears to detect a more committed population than the Pre-CFU assay.

The Pre-CFU assay is based on the finding that precursors to CFU-GM progenitors that do not grow in semisolid clonogenic assays with defined growth factors differentiate into CFU-GM following treatment in suspension phase, preferentially with the combination of IL-1 and IL-3. The combination of IL-1 + IL-3 generated the greatest number of secondary CFU-GM from 4-HC(terminal)/CD34+ human marrow compared with other cytokines including IL-3 alone. Additionally, in the primate model, significantly greater numbers of IL-1 + IL-3 responsive elements compared with IL-3–responsive elements were detected in the early hematopoietic recovery period following 5-FU myeloablution. These findings argue that in humans and primates, the combination of IL-1 plus IL-3 may stimulate an earlier differentiation compartment than IL-3 alone, or any of the other cytokines assayed. These observations are consistent with studies of Iscove and Yan indicating that murine Pre-CFU mix precursors proliferate preferentially in response to the combination of IL-1 + IL-3 relative to other cytokines.29 In our studies, as in those of Iscove and Yan, the combination of IL-6 + IL-3 was not as effective as IL-1 + IL-3 for inducing the proliferation of hematopoietic precursors. These findings are in contrast to descriptions of a synergistic effect of IL-6 with IL-3 in inducing proliferation of human and murine blast cell progenitors in the blast cell assay.11,29 These differences in observations may reflect differences in the culture conditions used or differences in the populations analyzed. In particular, in previous studies using the human blast cell assay, the populations used were less purified than the fractions used here, so that accessory cell activity possibly confounded interpretation of the culture outcomes.

The conclusion that the Pre-CFU assay detects very primitive hematopoietic precursors is based on several observations. First, IL-1 + IL-3 does not merely act by expanding residual CFU-GM progenitors, as large numbers of secondary CFU-GM were observed in individual experiments when there was a complete absence of D0 CFU-GM in CD34+ 4-HC(terminal) human BM, primate 5-FU–treated BM, and lin CD34+ human BM. Second, in the 5-FU–treated primate model, populations detected by the Pre-CFU assay recover from 5-FU treatment within 7 days of cytoreduction, substantially before recovery of CFU-GM. The rapidity of the recovery of IL-1 + IL-3 responsive precursors argues, in fact, that this population is closely related developmentally to the pluripotent stem cell spared

| Table 3. Effect of IL-1 + IL-3 Treatment on lin CD34+ Cells |
|---------------------------------|-----------------|-----------------|
|                                  | Before IL-1 + IL-3 Treatment | Following IL-1 + IL-3 Treatment |
| CD34+ %                         | 90%                                         | 10%                                         |
| CD33+ %                         | 5%                                           | 85%                                         |
| Morphology                       | Uniform small with mixed myeloid elements     | Majority monocytoid with mixed myeloid elements |
| D0 CFU-GM IL-1 + IL-3            | 9.4 ± 2.1                                     | 463.7 ± 140                                 |
| responsive secondary CFU-GM      | 463.7 ± 140                                    | 98.6 ± 30                                   |

D0 CFU-GM and secondary CFU values are means and standard error of three separate experiments. FACS analysis and Wright-Giemsa staining for morphology was performed on a single representative experiment. The difference between D0 CFU-GM and secondary CFU-GM was significant at P < .08 before IL-1 + IL-3 treatment and at P < .08 following IL-1 + IL-3 treatment.
CHARACTERIZATION OF HEMATOPOIETIC PRECURSORS

by 5-FU treatment. Third, the Pre-CFU assay was used to assess purification of a human precursor population, the lin CD34+ population, which is devoid of CFU-GM and is phenotypically similar to Pre-CFU populations defined previously using the LTBM system.16,20

Following the demonstration that IL-1 + IL-3 induces the differentiation of lin CD34+ precursors into CFU-GM progenitors, we examined whether IL-1 + IL-3 treatment resulted in expansion of the precursor population as well. Phenotypic analysis using Wright-Giemsa staining and FACS analysis for CD33+ and CD34+ proportion and functional analysis using the Pre-CFU assay was performed on lin CD34+ cells before and following 7 days of IL-1 + IL-3 treatment. The decline in Pre-CFU values and the increase in CD33+ and CD34+ cells following IL-1 plus IL-3 treatment indicate that this cytokine combination acts mainly to induce proliferation and differentiation of lin CD34+ cells without significant expansion of precursors to CFU-GM.

Currently, we are using the combination of FACS determination of CD34+CD33+SBA- cell number and the Pre-CFU assay to investigate a variety of culture conditions designed to expand hematopoietic precursor populations. The Pre-CFU assay cannot be used alone to accurately quantitate early precursors because the secondary CFU number can be confounded by differentiating cells that retain a proliferative response to IL-1 + IL-3. Observing an increase in total CD34+CD33+SBA- cells combined with an increase in the Pre-CFU value, however, would argue that a particular culture condition has resulted in expansion of early stem cells. Decline in the Pre-CFU value following a particular culture condition indicates that these conditions did not generate an expansion of precursor cells.

Currently, it is not clear whether the Pre-CFU assay detects actual reconstituting stem cells or merely a very early myeloid compartment. To address this issue, autologous BM and peripheral blood stem cell transplants comparing fractionated populations with high and low Pre-CFU assays are currently being performed. Correlation of engraftment parameters with Pre-CFU assay values will provide insight into the predictive value of this assay for measuring actual reconstituting stem cells. In addition, the Pre-CFU assay is being used to assess autologous BMT purging procedures, detection of residual hematopoietic precursor cells in aplastic marrow, and evaluation of congenital disorders of hematopoiesis. These studies should further enhance understanding the relationship of IL-1 + IL-3 responsive precursors to pluripotent stem cells, and should point the way to more accurate methods for in vitro assessment and manipulation of hematopoietic stem cells.

REFERENCES


Purification and partial characterization of a human hematopoietic precursor population

C Smith, C Gasparetto, N Collins, A Gillio, MO Muench, RJ O'Reilly and MA Moore

Updated information and services can be found at:
http://www.bloodjournal.org/content/77/10/2122.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml