Cytokine-Induced Selective Expansion and Maturation of Erythroid Versus Myeloid Progenitors From Purified Cord Blood Precursor Cells

By Hector Mayani, Wieslawa Dragowska, and Peter M. Lansdorp

To study the role of different cytokine combinations on the proliferation and differentiation of highly purified primitive progenitor cells, a serum-free liquid culture system was used in combination with phenotypic and functional analysis of the cells produced in culture. CD34+ CD45RA- CD71th cells, purified from umbilical cord blood by flow cytometry and cell sorting, were selected for this study because of their high content of clonogenic cells (34%), particularly multipotent progenitors (CFU-C, 12% of all cells). Four cytokine combinations were tested: (1) mast cell growth factor (MGF; a c-kit ligand) and interleukin-6 (IL-6); (2) MGF, IL-6, IL-3, and erythropoietin (Epo); (3) MGF, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL-3 fusion protein (FP), macrophage colony-stimulating factor (M-CSF), and granulocyte-CSF (G-CSF); and (4) MGF, IL-6, FP, M-CSF, G-CSF, and Epo. Maximum numbers of erythroid progenitors (BFU-E, up to 55-fold increase) and mature erythroid cells were observed in the presence of MGF, IL-6, IL-3, and Epo, whereas maximum levels of myeloid progenitors (CFU-C, up to 70-fold increase) and mature myeloid cells were found in cultures supplemented with MGF, IL-6, FP, M-CSF, and G-CSF. When MGF, IL-6, FP, M-CSF, G-CSF, and Epo were present, maximum levels of both erythroid and myeloid progenitors and their progeny were observed. These results indicate that specific cytokine combinations can act directly on primitive hematopoietic cells resulting in significant expansion of progenitor cell numbers and influencing their overall patterns of proliferation and differentiation. Furthermore, the observations presented in this study suggest that the cytokine combinations used were unable to bias lineage commitment of multipotent progenitors, but rather had a permissive effect on the development of lineage-restricted clonogenic cells.

MATURE BLOOD CELLS are derived from hematopoietic stem/progenitor cells (HSCs) that are typically present in specific (hematopoietic) tissues at very low frequencies (<0.1%). From the last 2 months of human prenatal life and during the entire postnatal life, the vast majority of HSCs are believed to reside in the bone marrow (BM). Interestingly, significant numbers of human HSCs can also be found in circulation; that is, umbilical cord blood, and adult peripheral blood. Indeed, recent studies indicate that both cord blood and peripheral blood can be used as sources of HSCs for allogeneic transplantation.5,6 Proliferation and differentiation of HSCs are regulated by a group of glycoproteins known as hematopoietic cytokines. These molecules are produced by a great variety of cells of both hematopoietic and nonhematopoietic origin and regulate HSC behavior via direct or indirect mechanisms.8 Despite an increased understanding of the biochemistry and molecular biology of these cytokines, their roles in the biology of primitive HSCs are not fully understood. In the present study, we have isolated a population of primitive hematopoietic cells from human cord blood that express high levels of the antigen CD34 and low or undetectable levels of the antigens CD45RA and CD71. By culturing these cells at low concentrations for 8 to 10 days in highly defined serum-free liquid cultures supplemented with various hematopoietic cytokines, we have achieved a significant expansion (up to 123-fold) of the CD34+ cell population, as well as myeloid (up to 70-fold), erythroid (up to 55-fold), and pluripotent (4-fold) progenitor cells. Interestingly, we found that the type of progenitor cells produced and the differentiation patterns of such primitive cells varied in response to the different cytokine combinations used.

MATERIALS AND METHODS

Cell separation. Cord blood cells, collected according to institutional guidelines, were obtained during normal full-term deliveries. Low-density cells (<1.077 g/mL) were isolated using Ficoll-Paque, washed twice in phosphate-buffered saline (PBS), resuspended in Hank’s HEPES-buffered salt solution containing 30% fetal calf serum (FCS) and 7.5% dimethyl sulfoxide (DMSO), aliquoted and frozen until used. Vials of frozen cells were rapidly thawed and slowly diluted with Iscove’s medium containing 30% FCS and 0.1 mg/mL DNA-se (type II-S, D4513; Sigma Chemical Co, St Louis, MO). Cells were then washed twice and resuspended in Hank’s HEPES-buffered salt solution containing 2% FCS and 0.1% sodium azide (HFN) for subsequent staining.

Cells were stained as previously described. Briefly, cells (107/mL) were incubated simultaneously with monoclonal antibodies (MoAbs) specific for CD34 (8G12 labeled with cyanine 5-succinimidyl ester [Cy5]), CD71 (OKT9 labeled with fluorescein isothiocyanate [FITC]), and CD45RA (8d2 labeled with phycoerytrin [PE], PI, and Cy5 excited at 20, 1, and 4 pg/mL, respectively, for 30 minutes at 4°C. Controls consisted of single-stained suspensions and three-color staining with anti-TNP-RPE instead of 8d2-RPE. Cells were then washed twice and resuspended in HFN containing 2 pg/mL propidium iodide (PI) before sorting. Cells were sorted on a FACStar Plus (Becton Dickinson, Mountain View, CA) equipped with a 5-W argon and a 30-mW helium neon laser. Specific fluorescence of FITC, RPE, and Cy5 excited at 488 nm (0.4 W) and 633 (30 mW), as well as forward and orthogonal light scatter signals, were used to establish sort windows. Cells were arbitrarily separated into fractions expressing low or undetectable levels of any of the antigens.
(eg, CD71<sup>hi</sup>) and cells expressing intermediate or high levels of antigen (eg, CD71<sup>hi</sup>), as described in the Results section.

Cell culture. Purified CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> cells were cultured in serum-free medium consisting of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with bovine serum albumin (2%), insulin (10 µg/mL), transferrin (200 µg/mL), 2-mercaptoethanol (10<sup>-3</sup> mol/L), low-density lipoprotein (40 µg/mL), and pen-strep (10<sup>5</sup> U and 50 µg/mL, respectively). The medium was supplemented with combinations of the following hematopoietic cytokines: mast cell growth factor or stem cell factor (MGF; 50 ng/mL), interleukin-6 (IL-6; 10 ng/mL), IL-3 (20 ng/mL), erythropoietin (Epo; 3 U/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL-3 fusion protein (FP; 20 ng/mL), macrophage colony-stimulating factor (M-CSF; 10 ng/mL), and granulocyte colony-stimulating factor (G-CSF; 21 ng/mL). MGF, IL-6, and FP were kindly provided by Dr. D. E. Williams (Immunex, Seattle, WA). IL-3, Epo, and G-CSF were provided by colleagues in our laboratory. M-CSF was a gift from Genetics Institute (Cambridge, MA). Control cultures contained no hematopoietic cytokines. Cells were cultured in 24-well tissue culture plates (Nunc, Kamstrup, Denmark) at 37°C, 5% CO<sub>2</sub>, at a concentration of 500 to 2,000 cells/well. After 8 to 10 days, cells were harvested from the wells, washed, counted in a hemocytometer using trypan blue and stained with MoAbs (as described above) and analyzed on a FACStar Plus.

Clonogenic assays. To determine their content of myeloid (CFU-C), erythroid (BFU-E), and multipotent (CFU-MIX) progenitors, CD34<sup>+</sup> cells were plated in semisolid cultures, as described previously, at a concentration of 100 cells/mL. The cultures were supplemented with agar-leukocyte-conditioned medium (10% vol/vol; Media Preparation Service, Terry Fox Laboratory, Vancouver, BC, Canada), Epo (3 U/mL), MGF (30 ng/mL), and FP (20 ng/mL). In some experiments, unseparated hematopoietic cells, harvested from liquid cultures after 8 days, were plated in semisolid cultures at a final concentration of 5,000 cells/mL.

RESULTS

Frequency of CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> cells. Cells expressing high levels of the antigen CD34 comprised 1.6% (range, 0.9% to 2.6%) of the total number of cord blood-derived low-density cells. Among them, cells expressing low or undetectable levels of the antigens CD45RA and CD71 (CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> cells) comprised 26.7% (range of 8.0% to 38.5%). CD34<sup>+</sup> CD45RA<sup>+</sup> CD71<sup>+</sup>, CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>+</sup>, and CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>hi</sup> cells comprised, on average, 25%, 16%, and 32%, respectively. A typical distribution of CD34<sup>+</sup> cells among low-density cells and phenotypic characterization of CD34<sup>+</sup> and CD34<sup>+</sup> cells with respect to CD45RA and CD71, as well as the sort windows used to isolate the progenitors used in this study, are shown in Fig 1.

Progenitor cell content in CD34<sup>+</sup> subpopulations. All four CD34<sup>+</sup> cell subpopulations mentioned above were plated in semisolid culture medium to determine their content of colony-forming cells. As shown in Table 1, 34% of the CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> cells were able to form hematopoietic colonies in methylcellulose after 14 days of culture. Thirty-nine percent of those clonogenic cells were myeloid progenitors, whereas erythroid and multipotential progenitors comprised 29% and 32%, respectively. CD34<sup>+</sup> CD45RA<sup>+</sup> CD71<sup>lo</sup>, and CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>hi</sup> cells had cloning efficiencies of 18% and 37%, respectively. The former consisted almost entirely of myeloid progenitors (90%), whereas the latter was enriched for erythroid progenitors (70%). CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> cells (cloning efficiency of 32%) contained significant levels of CFU-C (55%), BFU-E (35%), and CFU-MIX (10%). It is important to mention that the majority of BFU-E present in the CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> subpopulation gave rise to large erythroid colonies (more than three clusters), whereas a significant proportion of the BFU-E present in the other three subpopulations gave rise to small colonies (one to three clusters). Similarly, most of the CFU-C contained in the CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> subpopulation gave rise to larger myeloid colonies than those contained in the other subpopulations.

Cytokine-induced expansion and maturation of progenitor cells. Purified CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>lo</sup> cells were cultured in serum-free liquid cultures at concentrations of 500, 800, or 2,000 cells/well in the absence or in the presence of different cytokine combinations. For practical purposes, the absolute cell numbers after 8 to 10 days of culture have been normalized and are presented as per 1,000 input cells. When no cytokines were added to the cultures, the total number of cells present in the culture after 8 to 10 days was significantly decreased to 20% (7% to 30%) of the input levels (Table 2). Because of these low numbers, phenotypic analysis of these cells was not pursued. In cultures supplemented with MGF plus IL-6, there was a significant expansion of the total cell number (18-fold; Table 2). Approximately 40% of such cells expressed intermediate or high levels of the antigens CD45RA and CD71 (CD45RA<sup>+</sup> CD71<sup>hi</sup> cells). Another 40% were CD45RA<sup>lo</sup> CD71<sup>hi</sup>. CD34 was not expressed by any of the above subpopulations. The remaining 20% were still CD34<sup>+</sup> cells, with a mean absolute number of 3.2 × 10<sup>3</sup> cells (Table 3). This indicated a mean increment of three-fold in the number of CD34<sup>+</sup> cells after 8 to 10 days of culture. Most of the CD34<sup>+</sup> cells (65%) also expressed intermediate/high levels of CD45RA and CD71 (Table 3). Cells with the parental phenotype comprised less than 3%.

When cultures were supplemented with MGF, IL-6, IL-3, and Epo, a mean increment of 847 (448 to 1,375)-fold was observed in the total number of cells (Table 2). The majority of them expressed low/undetectable levels of CD45RA and intermediate or high levels of CD71 (Table 2, Fig 2). Only 3.4% of the cells expressed the antigen CD34; however, their mean absolute number was 28.7 × 10<sup>3</sup> (Table 3), which corresponded to a mean increment of 29 (6 to 32)-fold as compared with the input number of CD34<sup>+</sup> cells. The proportion of CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>+</sup> and CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>hi</sup> cells was about 50% each (Table 3 and Fig 2), with mean absolute numbers of 13.7 (2.4 to 15.7) and 13.2 (3.9 to 22.1) × 10<sup>3</sup> cells, respectively. CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>hi</sup> cells comprised less than 1%

Incubation of CD34<sup>+</sup> CD45RA<sup>lo</sup> CD71<sup>hi</sup> cells in the presence of MGF, IL-6, FP, M-CSF, and G-CSF resulted in a 673 (284 to 1,407)-fold increase in the total cell number after 8 to 10 days of culture (Table 2). Most of them (50% to 70%) expressed intermediate/high levels of both CD45RA
Fig 1. Selection of CD34+ CD45RA- CD71- cells from human umbilical cord blood by flow cytometry. (A) Selection of CD34+ cells, (B) correlated expression of CD45RA and CD71 of total cells, and (C) correlated expression of CD45RA and CD71 of CD34+ cells. The boxed area in (C) indicates the criteria used for selection of CD34+ CD45RA- CD71- cells among CD34+ cells. All dot plots are derived from low-density cells that are propidium iodide negative. Fluorescence is plotted on a log scale.

and CD71 (Table 2 and Fig 2). CD34+ cells comprised 5.7% of the total cell number. The absolute number of CD34+ cells was $42.3 \times 10^3$ (Table 3), which indicated a 42 (20 to 48)-fold increase when compared with the input number of CD34+ cells. The majority of these cells expressed both CD45RA and CD71 (Table 2 and Fig 2), and their mean absolute number was $32.3 (9.8$ to $34.7) \times 10^3$ (a mean 2.4-fold increase as compared with their number in cultures containing MGF, IL-6, IL-3, and Epo). On the other hand, the mean absolute number of CD34+ cells expressing low/undetectable levels of CD45RA and intermediate/high levels of CD71 was $7.94 (0.7$ to $9.8) \times 10^3$, which corresponded to 61% of their mean number in cultures supplemented with MGF, IL-6, IL-3, and Epo. CD34+ cells expressing low/undetectable levels of both CD45RA and CD71 comprised less than 1%.

In cultures supplemented with MGF, IL-6, FP, M-CSF, G-CSF, and Epo, a mean 1429 (648 to 2370)-fold increase in the total cell number was observed after 8 to 10 days (Table 2). CD45RA+ CD71+ and CD45RA- CD71- cells comprised 38% and 58%, respectively, whereas CD34+ cells accounted for 4% of the total cell number. The mean absolute number of the latter was $55.7 \times 10^3$ (Table 3), which indicates a 56 (14 to 123)-fold increase as compared with the input levels of CD34+ cells. Among these cells, 66% expressed intermediate/high levels of both CD45RA and CD71 (Table 3), with a mean absolute number of $34.2 (8.6$ to $41.4) \times 10^3$ (very similar to the mean total number observed in cultures containing MGF, IL-6, IL-3, and Epo). CD34+ CD45RA+ CD71- cells comprised 29% of the total number of CD34+ cells, and their mean absolute number was $14.6 (0.9$ to $17.3) \times 10^3$, similar to the one observed in cultures supplemented with MGF, IL-6, IL-3, and Epo.

In two of the experiments, the progenitor cell content after 8 days of culture was determined by culturing 5,000 unseparated cells in methylcellulose and correlating the number of progenitor cells detected in this condition with the total cell number observed in liquid cultures. As shown in Table 4, 60% to 64% of the progenitor cells present in cultures supplemented with MGF, IL-6, IL-3, and Epo were BFU-E, with absolute numbers of $2.41 \times 10^3$ and $3.34 \times 10^3$, respectively. This indicated increments of 31- and 55-fold, respectively, as compared with input values. CFU-C absolute numbers were $1.19 \times 10^3$ and $1.93 \times 10^3$, respectively, which indicated increments of 12- and 30-fold, respectively. CFU-MIX numbers were increased twofold and fourfold, respectively.

When liquid cultures were supplemented with MGF, IL-6, FP, M-CSF, and G-CSF, 72% to 78% of the progenitor cells observed were CFU-C. Their absolute numbers were $3.92 \times 10^3$ and $4.42 \times 10^3$, respectively (Table 4), indicating increments of 41- and 70-fold, respectively, as compared with input values. BFU-E were increased 12- and 23-fold, respectively; and CFU-MIX were increased 2- and 3.5-fold, respectively. In liquid cultures supplemented with MGF, IL-6, FP, M-CSF, G-CSF, and Epo, maximum levels of all three types of progenitors (BFU-E, CFU-C, and CFU-MIX) were observed (Table 4).

### Table 1. Growth of Umbilical Cord Blood CD34+ Subpopulations in Semisolid Cultures

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cloning Efficiency (%)</th>
<th>Type of Progenitor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+ CD45RA- CD71-</td>
<td>34 ± 4</td>
<td>BFU-E</td>
</tr>
<tr>
<td>CD34+ CD45RA- CD71-</td>
<td>32 ± 4</td>
<td>BFU-E</td>
</tr>
<tr>
<td>CD34+ CD45RA+ CD71+</td>
<td>18 ± 5</td>
<td>BFU-E</td>
</tr>
<tr>
<td>CD34+ CD45RA+ CD71+</td>
<td>32 ± 4</td>
<td>BFU-E</td>
</tr>
<tr>
<td>CD34+ CD45RA+ CD71-</td>
<td>37 ± 3</td>
<td>BFU-E</td>
</tr>
<tr>
<td>CD34+ CD45RA- CD71-</td>
<td>32 ± 4</td>
<td>BFU-E</td>
</tr>
</tbody>
</table>

Results represent mean ± SD from five experiments for CD34+ CD45RA+ CD71+ cells and three experiments for the other three subpopulations. Each experiment corresponds to a different cord blood. Cells of the indicated phenotype were plated at a concentration of 100 cells per dish (1 mL) and the colonies were scored on day 14.
IN VITRO EXPANSION OF CORD BLOOD PROGENITORS

that the majority of long-term culture initiating cells present significantly combinations.

No cytokines 0.21 (0.07-0.32)* ND ND ND
MFG + IL-6 18.2 (13-27) 39 ± 13 42 ± 15 20 ± 9.2
MFG + IL-6 + IL-3 + Epo 847 (448-1,375) 22 ± 14 76 ± 14 3.4 ± 1.7
MFG + IL-6 + FP + M-CSF + G-CSF 673 (284-1,407) 62 ± 10 34 ± 11 5.7 ± 2.1
MFG + IL-6 + FP + M-CSF + G-CSF + Epo 1,429 (648-2,370) 38 ± 14 58 ± 11 3.9 ± 2.4

Results represent mean ± SD from seven experiments, each one corresponding to a different cord blood, and are expressed as absolute cell numbers per 1,000 CD34+ CD45RA+ CD7+ input cells. Cells were harvested and analyzed after 8 to 10 days of culture.

Abbreviation: ND, not determined.

* Numbers in parentheses indicate range.

DISCUSSION

In the present study, we demonstrate the differential expansion in vitro of human erythropoiesis and/or myelopoiesis from a primitive cell population (CD34+ CD45RA+ CD7+) derived from cord blood. Such an expansion was achieved by culturing these cells in a defined serum-free liquid culture supplemented with different cytokine combinations.

CD34+ CD45RA+ CD7+ cells comprised 25% of the CD34+ population in cord blood. This subpopulation was highly enriched for progenitor cells. Indeed, their cloning efficiency (34% ± 4%) was significantly higher than the ones reported previously for CD34+ cells derived from BM10-14 or cord blood.15-19 Among the progenitor cells, all three major types (i.e., CFU-MIX, BFU-E, and CFU-C) were observed at significant levels, which indicates that this primitive subpopulation is still heterogeneous. However, it is noteworthy that pluripotential progenitors (CFU-MIX) were significantly increased, comprising one third of the total progenitor cell number. This observation and our previous report showing that the majority of long-term culture initiating cells present in BM are contained within CD34+ CD45RA+ CD7+ cells support the notion that cells with this phenotype are highly enriched for very primitive hematopoietic cells. Furthermore, the majority of BFU-E and CFU-C present in the CD34+ CD45RA+ CD7+ subpopulation gave rise to significantly larger colonies than the progenitors present in the CD34+ CD45RA+ CD7+ and CD34+ CD45RA+ CD7+ subpopulations, which again suggests that they indeed include primitive progenitor cells. Experiments regarding the functional characterization of the other CD34+ subpopulations, particularly CD34+ CD45RA+ CD7+ (90% CFU-C) and CD34+ CD45RA+ CD7+ (70% BFU-E), are currently in progress.

Hematopoietic progenitor and mature cell numbers were dramatically expanded when CD34+ CD45RA+ CD7+ cells were cultured for 8 to 10 days in serum-free liquid cultures supplemented with different cytokine combinations. It is noteworthy that some variability in the number of cells produced was observed between the different experiments. The intrinsic variability between the cord blood samples as well as differences in the numbers of cells used to initiate the cultures (ranging from 500 to 2,000) and/or the days at which the cells were harvested (ranging from day 8 to day 10) could account for these differences. Nevertheless, the overall proliferation and differentiation patterns observed in response to the different cytokine combinations were similar in each experiment.

When no cytokines were added to the culture, there was a decrease in the total cell number, which indicated that the cord blood progenitors, similar to their previously described counterparts in BM, require hematopoietic cytokines for survival. In the presence of cytokine combinations, the total numbers of nucleated cells as well as CD34+ cells were increased. The relative proportion and absolute number of mature cells and the different CD34+ subpopulations depended on the cytokine combination used. When the cytokine combination included factors that preferentially influ-

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Total Cell No. (×10^3)</th>
<th>CD45RA+ CD7+ (%)</th>
<th>CD45RA+ CD7+ (%)</th>
<th>CD34+</th>
</tr>
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<tr>
<td>No cytokines</td>
<td>0.21 (0.07-0.32)*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MFG + IL-6</td>
<td>18.2 (13-27)</td>
<td>39 ± 13</td>
<td>42 ± 15</td>
<td>20 ± 9.2</td>
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<tr>
<td>MFG + IL-6 + IL-3 + Epo</td>
<td>847 (448-1,375)</td>
<td>22 ± 14</td>
<td>76 ± 14</td>
<td>3.4 ± 1.7</td>
</tr>
<tr>
<td>MFG + IL-6 + FP + M-CSF + G-CSF</td>
<td>673 (284-1,407)</td>
<td>62 ± 10</td>
<td>34 ± 11</td>
<td>5.7 ± 2.1</td>
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<tr>
<td>MFG + IL-6 + FP + M-CSF + G-CSF + Epo</td>
<td>1,429 (648-2,370)</td>
<td>38 ± 14</td>
<td>58 ± 11</td>
<td>3.9 ± 2.4</td>
</tr>
</tbody>
</table>

Results represent mean ± SD from seven experiments, each one corresponding to a different cord blood, and are expressed as absolute cell numbers per 1,000 CD34+ CD45RA+ CD7+ input cells. Cells were harvested and analyzed after 8 to 10 days of culture.

Abbreviation: ND, not determined.

* Numbers in parentheses indicate range.

Table 3. Total Number and Phenotype of CD34+ Cells Developed in Umbilical Cord Blood-Derived Serum-Free Liquid Cultures

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Total Cell No. (×10^3)</th>
<th>CD34+ CD45RA+ CD7+ (%)</th>
<th>CD34+ CD45RA+ CD7+ (%)</th>
<th>CD34+</th>
</tr>
</thead>
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<td>No cytokines</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
<td>MFG + IL-6</td>
<td>3.2 (1.3-5.8)*</td>
<td>65 ± 15</td>
<td>29 ± 16</td>
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<tr>
<td>MFG + IL-6 + IL-3 + Epo</td>
<td>28.7 (6.5-31.7)</td>
<td>49 ± 17</td>
<td>46 ± 16</td>
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<tr>
<td>MFG + IL-6 + FP + M-CSF + G-CSF</td>
<td>42.3 (19.8-48.4)</td>
<td>81 ± 11</td>
<td>19 ± 12</td>
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<tr>
<td>MFG + IL-6 + FP + M-CSF + G-CSF + Epo</td>
<td>55.7 (14.0-123.2)</td>
<td>66 ± 12</td>
<td>29 ± 15</td>
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</table>

Results represent mean ± SD from seven experiments, each one corresponding to a different cord blood, and are expressed as absolute cell numbers per 1,000 CD34+ CD45RA+ CD7+ input cells. Cells were harvested and analyzed after 8 to 10 days of culture.

Abbreviation: ND, not determined.

* Numbers in parentheses indicate range.
Fig 2. Differentiation patterns of cord blood–derived primitive hematopoietic cells in response to different cytokine combinations. Phenotypic characterization of cells in serum-free cultures initiated with purified CD34− CD45RA− CD71lo cells and supplemented with either MGF, IL-6, IL-3, and Epo or MGF, IL-6, FP, M-CSF, and G-CSF. All dot plots are from a single representative experiment; cells were harvested after 10 days of culture. The thresholds set to discriminate between CD45RA+ and CD45RA− cells and CD71lo and CD71high cells were those used to create the boxed area in Fig 1C.

ence erythropoiesis (IL-3 and Epo) together with synergistic cytokines (MGF and IL-6), there was a preferential expansion of erythroid progenitor (CD34− CD45RA− CD71+/− BFU-E) and mature (CD34− CD45RA− CD71+) cells. On the other hand, when factors affecting primarily myelopoiesis (GM-CSF/IL-3 fusion protein, M-CSF, and G-CSF) were combined with MGF and IL-6, there was a preferential expansion of myeloid progenitor (CD34− CD45RA−

### Table 4. Progenitor Cell Numbers in Umbilical Cord Blood–Derived Serum-Free Liquid Cultures

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Total No. of Progenitor Cells</th>
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<tbody>
<tr>
<td></td>
<td>CFU-C</td>
<td>Exp1</td>
<td>Exp2</td>
<td>Exp1</td>
<td>Exp2</td>
<td>Exp1</td>
<td>Exp2</td>
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<td>(Exp1)</td>
<td>(Exp2)</td>
<td>(Exp1)</td>
<td>(Exp2)</td>
<td>(Exp1)</td>
<td>(Exp2)</td>
<td>(Exp1)</td>
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<tr>
<td>MGF + IL-6 + IL-3 + Epo</td>
<td>1,190</td>
<td>1,930</td>
<td>2,410</td>
<td>3,340</td>
<td>170</td>
<td>270</td>
<td>3,770</td>
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<tr>
<td>MGF + IL-6 + FP + M-CSF + G-CSF</td>
<td>3,920</td>
<td>4,420</td>
<td>980</td>
<td>1,430</td>
<td>180</td>
<td>240</td>
<td>5,080</td>
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<tr>
<td>MGF + IL-6 + FP + M-CSF + G-CSF + Epo</td>
<td>3,670</td>
<td>4,370</td>
<td>2,260</td>
<td>3,410</td>
<td>200</td>
<td>270</td>
<td>6,130</td>
</tr>
</tbody>
</table>

Results represent absolute progenitor cell numbers from two experiments, each one corresponding to a different cord blood. Cells were harvested after 8 days of culture from serum-free liquid cultures, supplemented with the indicated cytokine combination. Without further separation, cells were plated in semisolid cultures at a concentration of 5,000 cells/mL and colonies were scored on day 14. Total progenitor cell numbers were obtained based on the number of progenitors per 5,000 cells plated in methylcellulose and the total cell numbers in liquid cultures. Numbers in parentheses indicate progenitor cell numbers at day 0.
CD71+ (CFU-C) and mature (CD34+ CD45RA+ CD71+) cells. When all the above factors were added together, maximum numbers of both erythroid and myeloid cells were obtained. These results indicate that the proliferation and differentiation patterns of very primitive hematopoietic cells can be directly influenced by hematopoietic cytokines. The actual mechanisms operating in this system are not resolved as yet. Our observation that addition of Epo to culture conditions highly supportive of myeloid cells (MGF, IL-6, FP, M-CSF, G-CSF) did not alter the number of these cells while it increased the number of erythroid cells suggests that the cytokine environment acts in a permissive manner in the expansion and maturation of distinct cell lineages rather than in a deterministic way. However, this observation must be interpreted with caution because the cell population analyzed in this study, although primitive, is still heterogeneous and the approach followed in this study does not permit us to assess the effects of hematopoietic cytokines on uncommitted stem cells. We are currently addressing this issue by assessing the effects of cytokine combinations on single CD34+ CD45RA+ CD71+ cells.

In vitro expansion of cord blood progenitor cells has been previously documented. Broxmeyer et al showed a 2- to 16-fold increase in progenitor cell number in cultures of unseparated cord blood cells containing MGF in combination with a second cytokine. Migliaccio et al reported a 2- to 20-fold increase in cultures of CD34+ soybean agglutinin-negative cells. Interestingly, these investigators found that most of the expanded progenitors were BFU-E and CFU-E when MGF was added in combination with Epo. In contrast, when MGF was combined with G-CSF, almost all of the progenitors were CFU-C. Abbud et al documented a 23- and 30-fold increase in BFU-E and CFU-C numbers, respectively, after culturing cord blood CD34+ 4-HC- cells for 7 days in suspension cultures supplemented with IL-1 and IL-3. In the present study, we have obtained significantly higher increments in progenitor cell numbers. This could be due to the fact that the cytokine combinations and/or the culture conditions used here were more effective in expanding progenitor cells than those used in the above studies. Alternatively, these differences could be explained by the fact that we started our cultures with a more defined or perhaps less compromised (ie, not treated with 4-HC) cell population that may have a higher proliferative potential.

In vitro expansion and maturation of hematopoietic progenitor cells might be of particular relevance in the treatment of patients after hematopoietic transplantation. Haylock et al have recently postulated that the period of hematopoietic recovery (ie, neutrophil and platelet counts and hemoglobin levels) after transplant might be significantly shortened if expanded progenitor and postprogenitor cell populations are infused to the patients. These investigators achieved a 1,324-fold increase in nucleated cell number and a 66-fold increase in the number of CFU-C from peripheral blood-derived CD34+ cells cultured in liquid culture supplemented with a combination of six different cytokines (IL-1, IL-3, IL-6, MGF, GM-CSF, G-CSF). Our results are comparable with the ones presented in their report and suggest that similar approaches could be followed using cord blood-derived hematopoietic cells. For such applications, information on the in vivo function of in vitro-expanded progenitors as well as the effects of such an expansion on the cells required for long-term hematopoietic reconstitution is highly desirable. With the use of appropriate animal models, such information could be obtained in the near future.

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Cytokine-induced selective expansion and maturation of erythroid versus myeloid progenitors from purified cord blood precursor cells

H Mayani, W Dragowska and PM Lansdorp