Synergism Between Erythropoietin and Interleukin-3 in the Induction of Hematopoietic Stem Cell Proliferation and Erythroid Burst Colony Formation

By Giovanni Migliaccio, Anna Rita Migliaccio, and Jan W.M. Visser

The influence of recombinant erythropoietin (Ep) and interleukin-3 (IL-3) on the proliferation and differentiation of murine hematopoietic stem and progenitor cells was investigated in serum-deprived cultures. The differentiation of progenitor cells, purified by collecting blast cell colonies from spleen cell cultures of 5-fluorouracil-treated mice, was evaluated by scoring the number and type of colonies appearing after eight days in semisolid culture. IL-3 induced the formation of both erythroid and granulocyte-macrophage colonies in a concentration-dependent fashion and the plateau being reached at 300 U/mL. However, concentrations of IL-3 alone that had little or no effect (≤10 U/mL) induced maximal numbers of erythroid bursts in the presence of Ep (1.5 IU/mL). In the presence of Ep alone, no colonies were seen. Proliferation of quiescent hematopoietic stem cells, purified by cell sorting and evaluated by spleen colony assay (CFU-S), was investigated by measuring the total cell number and CFU-S content.

Erythropoietin (Ep), a 34,000 dalton glycoprotein produced by the kidney, is the hormone responsible for the regulation of erythropoiesis in mammals. This regulatory role has been demonstrated both in vivo and in vitro. Perturbations of the plasma concentrations of Ep in vivo result in rapid changes in the numbers of erythroblasts. Furthermore, committed erythroid progenitors (colony-forming unit, erythroid; CFU-E) have Ep receptors on their surface and require the presence of the hormone to survive in vivo, to proliferate in vitro, and to activate the expression of erythroid-specific proteins, such as glycophorin and globin chains. More primitive erythroid progenitor cells (burst-forming unit, erythroid; BFU-E) or mixed-colony-forming cells (CFU-Mix or CFU-GEMM) and spleen colony-forming cells (CFU-S) require the presence of interleukin-3 (IL-3), a 30,000 dalton glycoprotein produced by the kidney, to survive and proliferate in vitro.

Several observations support the hypothesis that Ep may act directly on hematopoietic stem and progenitor cells: (1) mice treated with cytotoxic drugs will recover erythropoiesis more rapidly if repeatedly injected with Ep; (2) after Ep injection, the proportion of 3H-thymidine-sensitive BFU-E increases; (3) Ep preincubation increases the ratio between BFU-E and granulocyte-macrophage (GM) progenitors in culture of total bone marrow; (4) addition of the hormone is required for the generation of CFU-E in long-term bone marrow culture. On the other hand, delayed addition of the hormone, or its complete omission, has little, or no effect on the BFU-E cloning efficiency in serum-deprived culture of total bone marrow cells supplemented with IL-3.

In order to clarify the regulatory role exerted by Ep in the early phases of erythropoiesis, as well as its possible interaction with IL-3, we have investigated the effect of recombinant human Ep on the proliferation and differentiation of IL-3-stimulated purified progenitor cells cultured in the absence of fetal bovine serum (FBS). Particular attention was given to the dose of IL-3 used. The results indicate a synergism between Ep and IL-3 in the induction both of proliferation and differentiation of hematopoietic stem and progenitor cells.

MATERIALS AND METHODS

Growth factors. Spleen conditioned medium (SCM) was the supernatant of a serum-free culture of concanavalin A-stimulated murine splenocytes.

Pure natural murine IL-3 was kindly provided by Dr. J. Ihle. One unit was defined as the amount of the factor that gives 50% of the area of the FDC-P1 cell line. Pure recombinant murine IL-3 (rIL-3) was kindly provided by Dr. J.J. Mermod (Biogen, Geneva, Switzerland). One unit was defined as the amount of the factor that induces half-maximal proliferation of cells of the DA2 cell line. Pure recombinant human Ep (specific activity 160,000 U/mg of protein) was kindly provided by Dr. J.C. Egie (Amgen, Thousand Oaks, CA).

The amounts of each factor that induced the maximal number of colonies in serum-deprived cultures of total bone marrow were 5% (vol/vol) for SCM, 100 and 10 U/mL for IL-3 and rIL-3, respectively, and 1.5 IU/mL for recombinant Ep.

Mice. Either DBA/2 or C57BL/6 (Charles River, Calco, Italy) or BC3 (TNO, Rijswijk, The Netherlands) females (10 to 12 g) were used. BC mice (C57BL/6 x C3H F1, hybrids) were bred under specific pathogen-free conditions in order to facilitate the day-12 CFU-S assay (see below). Mice were provided with sterilized pelleted food and sterilized, acidified water ad libitum.

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**Culture medium for hematopoietic colony assay.** The semisolid medium contained the following components in Iscove's modified Dulbecco's medium (1%DMEM): methicellulose (0.8%, final concentration), β-mercaptoethanol (7.5 × 10⁻³ mol/L), and either FBS (40%, vol/vol) or a mixture of FBS-replacing components: deionized bovine serum albumin (BSA; fraction V, Sigma, St Louis) and BSA-adsorbed cholesterol (Sigma) (final concentration 2 × 10⁻⁴ mol/L for both), iron saturated human transferrin (Behring, Marburg, FRG) (9 × 10⁻⁶ mol/L), insulin (Sigma) (1.7 × 10⁻⁴ mol/L), nucleosides (10 μg/mL each), hemin (Sigma) (10⁻³ mol/L), sodium pyruvate (10⁻⁴ mol/L), and L-glutamine (2 × 10⁻³ mol/L) as reported.

For liquid suspension cultures, the same components were used except methicellulose was replaced by IMDM.

**Purification and culture of hematopoietic progenitors.** Murine progenitors were purified by collecting blast cell colonies (bcc) in primary cultures of splenocytes from 5-fluorouracil (5-FU)-treated mice.25 DBA2 mice injected intravenously (IV) with 5-FU in saline (150 mg/kg body weight), were killed four days later and the spleens were removed aseptically. Single cells in suspension (2 × 10⁵ cells/mL) were plated in 35-mm dishes containing 1 mL of the semisolid culture medium described above. The cultures were supplemented either with SCM (5%, vol/vol) or nIL-3 (10 U/mL). After seven days of incubation at 37°C in 5% CO₂ in air (−100% humidity), formation of bcc was detected (20 to 30 bcc/plate). Individual colonies were collected by means of a thin Pasteur pipette and pooled. The pool of bcc-derived cells was washed twice with fresh culture medium and plated at low cell concentration (250 to 500 cells/mL) in secondary serum-deprived cultures. Subsequent colonies were scored after eight days of incubation.

**Scoring criteria.** Hematopoietic colonies, scored at day eight of culture unless otherwise stated, were typed as follows: erythroid cells/mL in secondary serum-deprived cultures. Subsequent cob- colonies composed of ≤50 cells were scored as clusters. In some experiments, the frequency of progenitor cells in colonies was analyzed by May-Grunwald/Giemsa staining to confirm the identity of their cell type.

**Purification and culture of hematopoietic stem cells.** Stem cells were purified as described previously.26 Briefly, adult mouse bone marrow cells were collected by flushing the femurs with Hank's balanced salt solution (HBSS; Laboratories Eurobio, Paris) buffered at pH 6.7 with HEPES (10 mmol/L; Merck, Rahway, NJ) and provided with penicillin (10 U/mL) and streptomycin (0.1 mg/mL). The cell suspension was filtered through a nylon sieve. Subsequently, the cells were centrifuged (400 g for ten minutes at 4°C) in a discontinuous metrizamide (Nijegaard, Oslo) density gradient in the presence of wheat-germ agglutinin, which was bound to fluorescein isothiocyanate (WGA-FITC; 1 μg/10 mL metrizamide solution; Polysciences, Inc, Warrington, PA). The low density cells were washed once and analyzed using a light-activated cell sorter (FACS II; Becton Dickinson, Sunnyvale, CA). Cells with medium and high WGA-FITC fluorescence, medium forward, and low perpendicular light scatter intensities were sorted. Subsequently, WGA-FITC was removed from the sorted cells by incubation with an isotonic solution of N-acetyl-D-glucosamide (0.2 mol/L; Polysciences). The cells were then labeled with anti-H-2K-biotin and avidin-FITC (Becton Dickinson) and analyzed on the FACS II again. Two subpopulations of cells, dull and bright, were distinguished using H-2K marker. The brightly fluorescent subpopulation was sorted and either analyzed for DNA content, or the cells were injected into irradiated syngeneic recipients. In some experiments, the frequency of progenitor cells in the sorted fraction was also evaluated.

**CFU-S assay.** C57 mice were lethally irradiated using a ¹³¹Cs gamma-cell-20 small animal irradiator (Atomic Energy of Canada). The irradiation unit contained twin ¹³¹Cs gamma-ray sources, one located above and one below the irradiation compartment. The dose rate at the position of the mice was 0.9 Gy/min. Irradiated mice received a total dose of 9.0 Gy.

Aliquots of 0.5 mL containing either 3 × 10⁴ unseparated or 3 × 10⁵ sorted and cultured cells were injected via the tail vein immediately following the irradiation. Groups of recipients were killed at day 8 or 12, their spleens were fixed in Tellemyczsky's solution, and macroscopic colonies were counted. The spleens of irradiated mice that did not receive cells contained less than one colony per ten spleens.

**Flow cytometry.** The number and the DNA histogram of sorted cells was analyzed before and after the liquid culture using a slightly modified FACS II light-activated cell sorter (Becton Dickinson). The argon ion laser was set at 500 mW, 488 nm. Sheath fluid consisted of HBSS. Cells were sorted in glass tubes that were rinsed with HBSS containing 5% FBS before and after the sorting. DNA histograms were obtained by using propidium iodide staining as described by Taylor.28 Cell counts were performed by mixing the cell suspensions with known numbers of fluorescent beads and by using the FACS while measuring DNA histograms.

**RESULTS**

**Effect of nIL-3 and recombinant Ep on the formation of erythroid bursts and GM colonies in serum-deprived cultures of total bone marrow cells.** The mixture of nutrients used to replace FBS resulted in a cloning efficiency of 33 erythroid bursts and 170 GM colonies/5 × 10⁴ cells in cultures of total bone marrow stimulated with SCM (Fig 1). The addition of nIL-3 allowed the detection of erythroid bursts and of GM colonies in a concentration-dependent fashion (Fig 1). For both types of colonies, half maximal and maximal cloning efficiency were observed at nIL-3 doses of 20 and ~100 U/mL, respectively. The addition of Ep (1.5...
2.5 - SCM

Fig 3. Natural IL-3 concentration/response curve on erythroid bursts in serum-free cultures of bcc-derived cells in the presence (●) or absence (○) of Ep (1.5 IU/mL). The number of erythroid bursts detected in cultures stimulated with SCM is reported for comparison and is similar to that observed in FBS-supplemented cultures. No GM colonies were detected in IL-3-stimulated cultures while 43 ± 6 GM colonies were observed in the presence of SCM.

Fig 2. Dependence of total colony numbers (erythroid bursts + GM colonies + clusters) on the amount of plated bcc-derived cells in serum-deprived cultures stimulated with SCM (5%, vol/vol). (Mean values from a single experiment performed in triplicate.)

to 2 IU/mL) did not affect the number of GM colonies but increased twofold the number of erythroid bursts detected. No significant changes in the nIL-3 concentration/response curve were observed in the presence of Ep. These results are similar to those reported by Goodman et al.12

Purification of hematopoietic progenitors from bcc. Bcc-derived cells cultured under serum-deprived conditions in the presence of SCM (5%, vol/vol), gave rise to erythroid bursts, GM colonies and clusters of mast cell-like elements with a cloning efficiency of 25% to 50% (Fig 2). Twenty percent of erythroid bursts also contained G and M elements. The secondary cloning efficiency was directly proportional to the number of cells plated (Fig 2). In the absence of SCM, no colony formation was seen (Fig 3).

Effect of nIL-3 and rIL-3 on colony formation from bcc-derived progenitor cells. To determine if the colonies detected in nIL-3-stimulated cultures of total bone marrow were dependent on factors released in the dish by accessory cells, a series of experiments were performed to examine the effect of IL-3 on the formation of colonies by progenitor cells from bcc.

Formation of hematopoietic colonies was not observed in cultures of bcc-derived progenitor cells supplemented with concentrations of IL-3 (10 to 100 U/mL), which induced maximal colony formation in cultures of total bone marrow cells (Fig 3). Colony formation also was not observed in cultures stimulated with only Ep. However, in cultures to which both nIL-3 and Ep were added, pure erythroid bursts were seen in numbers nearly equal to the SCM-stimulated controls (Fig 3).

The availability of rIL-3 allowed us to investigate the effect of higher IL-3 concentrations on the induction of colony formation from bcc-derived progenitor cells. As units of activity were defined differently for nIL-3 and rIL-3 (see Materials and Methods), the concentration/response relationships of the two sources of growth factor were compared in serum-deprived culture of unfractinated bone marrow or of progenitor cells derived from bcc (Fig 4). In both cases,
rIL-3 was found to be ten times more active than the natural material (1 unit of rIL-3 = 10 units of nIL-3).

In cultures of bcc-derived cells, rIL-3 gave rise to mixed erythroid and GM colonies in a concentration-dependent fashion (Fig 5). Colonies were detected with concentrations of rIL-3 ≥ 30 U/mL (corresponding to 300 U/mL of nIL-3). The addition of Ep increased the number of mixed-cell colonies detected at high rIL-3 concentrations and allowed the detection of pure erythroid bursts at rIL-3 concentrations as low as 0.3 U/mL. (Fig 5).

The role of Ep in the early phases of erythroid differentiation was further evaluated either by adding Ep during the formation of bcc (Table 1) or delaying its addition in the secondary cultures until day 3 (Table 2). Addition of Ep to primary bcc cultures allowed the formation of erythroid progenitors that generated small Ep-dependent erythroid colonies (200 to 300 cells per colony) at day 3 or 4 in secondary cultures (Table 1). If the addition of Ep to IL-3–supplemented cultures of bcc-derived cells was delayed to day 3 or 4, mature erythroid colonies now formed on days 12 to 15. These colonies were large (~3 mm in size, 1.5 × 10³ cells per colony) and composed of ≥99% erythroblasts (Table 2). These results suggest that Ep and IL-3 not only act cooperatively in inducing erythroid differentiation, but also that they must be present simultaneously. For this reason we investigated the effect of IL-3 and Ep on the induction of proliferation of hematopoietic stem cells purified by cell sorting.

Purification of hematopoietic stem cells by cell sorting. The purification process yielded CFU-S enrichment factors of 127 and 245 for day 8 and day 12 CFU-S, respectively (Table 3). The DNA histogram of the sorted cells indicated that almost all of these cells were in G0 or G1 (Fig 6A, Table 4). The frequency of BFU-E in the purified fraction was 4%. CFU-E, if present, had a frequency <0.3% (Table 3). The morphology of ≥85% of the sorted cells, was that of undifferentiated blasts.26

Effect of recombinant Ep and IL-3 on the proliferation of purified stem cells in serum-deprived cultures. Purified stem cells were induced to proliferate in serum-deprived culture by addition of Ep and/or rIL-3. Two rIL-3 concentrations were investigated: 400 and 10 U/mL.

Very few cells, and no detectable CFU-S, survived 20 hours of suspension culture in the absence of stimulus (Table 5). A slightly higher proportion of cells survived 20 hours if stimulated only with 10 units of rIL-3 or Ep. The CFU-S content in these cultures was also low and, in particular, almost no day 12 CFU-S survived with Ep only (Table 5).

### Table 1. Erythroid Bursts and GM Colonies in Cultures of bcc-Derived Cells (250 Cells per Culture) Primed With Ep During the Primary Culture

<table>
<thead>
<tr>
<th>No. of Colonies/Dish</th>
<th>Stimulus</th>
<th>Day 3-4</th>
<th>Day 8</th>
<th>GM Day 8</th>
<th>Clusters Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ep</td>
<td>5 ± 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCM</td>
<td>0 11 ± 3</td>
<td>133 ± 6</td>
<td>53 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 U/mL nIL-3</td>
<td>0 0 0</td>
<td>40 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 U/mL nIL-3 + Ep</td>
<td>5 ± 1</td>
<td>10 ± 1</td>
<td>47 ± 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bcc have been collected from serum-free cultures of splenic cells (2 × 10⁶ cells/mL) stimulated with SCM (5%, vol/vol) and Ep (1.5 U/mL). Mean ± SD of three separate experiments performed in duplicate.

### Table 2. Erythroid Bursts and GM Colonies in Cultures of bcc-Derived Cells in Delayed Ep Addition Experiments (250 Cells per Dish)

<table>
<thead>
<tr>
<th>No. of Colonies per Dish</th>
<th>Stimulus</th>
<th>Day 3-4</th>
<th>Day 8</th>
<th>GM Day 8</th>
<th>Clusters Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ep</td>
<td>0 0 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SCM</td>
<td>0 7 ± 1</td>
<td>60 ± 6</td>
<td>39 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 U/mL nIL-3</td>
<td>0 0 0</td>
<td>29 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 U/mL nIL-3 + Ep</td>
<td>0 7 ± 1</td>
<td>20 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 U/mL nIL-3 + Ep*</td>
<td>0 0 0</td>
<td>39 ± 8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bcc have been collected from serum-free cultures of splenic cells (2 × 10⁶ cells/mL) stimulated with SCM (5%, vol/vol). Mean ± SD of three experiments performed in duplicate.

*Ep was added at day 3 of culture. When the plates were scored at day 12 to 15, 3-mm pure BFU-E derived colonies were detected in these dishes (1.5 × 10³ cells/colony, ≥99% erythroblasts). No increase in colony number was observed in all the other culture conditions.
Sixty percent of purified cells survived for 20 hours in the presence of 400 units of rIL-3. These cells were composed both of day-8 (71% of the input) and day-12 (41% of the input) CFU-S (Table 5). Eighteen percent of them were in the S + G2 + M phase of the cell cycle (Table 4). However, the DNA histogram (Fig 7) indicated that the cells did not enter the cycle as a synchronized cohort and almost none of them reached G2-M phase at 20 hours (Fig 7). The total cell number per culture did not increase at 20 hours (Table 5). At 48 hours, the percentage of cells in the S phase increased to 38% (Table 4, Fig 6B). An increase in the total cell number was also detectable (Table 4). Therefore, cell division occurred. However, this cell proliferation did not result in increased CFU-S numbers; on the contrary, a decrease in day-12 CFU-S was observed (Table 5).

In cultures stimulated with both rIL-3 (10 U/mL) and Ep, 42% of the cells survived at 20 hours (Table 5). These cells were composed of both day-8 (41% of the input) and day-12 (21% of the input) CFU-S (Table 5). Ten percent of these cells were in the S + G2 + M phase of the cell cycle at 20 hours and an increase in cell number was also detectable at 48 hours (Table 5). These values, although lower than those obtained with 400 units of rIL-3, are all higher than the sum of the corresponding values obtained with Ep or a low concentration of IL-3 alone (Tables 4 and 5). Addition of Ep did not affect those cultures stimulated with the higher concentration of IL-3.

**DISCUSSION**

rIL-3 (1 to 10 U/mL) and nIL-3 (10 to 100 U/mL) induced formation of erythroid bursts and GM colonies in serum-free cultures of unfractionated bone marrow cells (Figs 1 and 4). The biological activity of the bacterially expressed rIL-3 was equivalent to that of nIL-3 (Fig 4). The different concentrations at which the two hormones were active may be due to the different definitions of units of the two preparations (see Materials and Methods).

rIL-3 (30 to 300 U/mL) induced formation of mixed erythroid and GM colonies also in cultures of progenitor cells purified by collecting bcc from splenic cultures of 5-FU–treated animals (Fig 5). The concentration of rIL-3 required for maximal cloning efficiency was tenfold higher than that required in cultures of total bone marrow cells (Fig 3). The colonies observed in these cultures are likely induced by rIL-3 through a direct action on the progenitor cells. In fact, the frequency of progenitor cells in the bcc-derived cell population (25% to 50%) (Fig 2), the lack of endogenous colonies in these cultures (Figs 3 and 5), and the direct proportionality of the colony frequency to the number of bcc-derived cells plated (Fig 2), suggest that significant amounts of growth factors are not released in these cultures.

Similar results were reported by Suda et al, who found that nIL-3 (400 U/mL) stimulated colonies composed of neutrophils, macrophages, eosinophils, and megakaryocytes in serum-deprived cultures of single progenitor cells. The Ep seemed to be required by the erythroid lineage. However, since the frequency of mixed erythroid colonies is seven to 14 colonies per 500 bcc-derived cells, it is unlikely to detect IL-3–dependent mixed erythroid colonies in single cell culture experiments.

Pure erythroid bursts, in number equivalent to the SCM-stimulated controls, were observed when concentrations of nIL-3 (10 to 100 U/mL) or rIL-3 (0.3 to 10 U/mL), which
Table 4. Analysis of DNA Histogram of Sorted Bone Marrow Cells Cultured Under Serum-Deprived Conditions

<table>
<thead>
<tr>
<th>Time in Culture</th>
<th>Debris/ (Debris + Cells)</th>
<th>(G_0 + G_1)/Total Cells in Culture</th>
<th>(S + G_2 + M)/Total Cells in Culture</th>
<th>S + G_2 + M (% of 0 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h None</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20 h None</td>
<td>93</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>10 U r IL-3</td>
<td>83</td>
<td>83</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>400 U r IL-3</td>
<td>41</td>
<td>71</td>
<td>29</td>
<td>18</td>
</tr>
<tr>
<td>Ep</td>
<td>82</td>
<td>89</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Ep + 10 U r IL-3</td>
<td>56</td>
<td>77</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>Ep + 400 U r IL-3</td>
<td>43</td>
<td>75</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>48 h None</td>
<td>94</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>10 U r IL-3</td>
<td>71</td>
<td>64</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>400 U r IL-3</td>
<td>33</td>
<td>55</td>
<td>45</td>
<td>38</td>
</tr>
<tr>
<td>Ep</td>
<td>89</td>
<td>64</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>Ep + 10 U r IL-3</td>
<td>57</td>
<td>65</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>Ep + 400 U r IL-3</td>
<td>32</td>
<td>54</td>
<td>46</td>
<td>34</td>
</tr>
</tbody>
</table>

Abbreviation: NE, below the detectable level.

The same experiment is presented in Table 5. Similar results were obtained in four other experiments.

were unable to sustain formation of hematopoietic colonies from bcc-derived cells, were complemented by Ep (Figs 3 and 5). Similarly, formation of granulocyte and macrophage colonies was induced under the same IL-3 concentrations by addition of G- or M-CSF, respectively (data not shown).

Ep and low concentrations of IL-3 might act at the same time on progenitor cells to induce erythroid differentiation since erythroid bursts were observed at 12 to 15 days instead of the usual eight days of culture if the addition of Ep was delayed by three to four days in cultures of bcc-derived cells supplemented with 10 U/mL of nIL-3 (Table 2). Furthermore, the presence of Ep during the formation of bcc allowed the detection of CFU-E in the subsequent secondary culture (Table 1). This raises the possibility that Ep and IL-3 may exert a cooperative effect in the control of the cell cycle.

WGA* and H-2K* marrow cells, selected by cell sorter, represent a suitable source of stem and progenitor cells to study the cell cycle. In fact all of them are in the G_0 or G_1 phase of the cycle (Fig 6A) and can be prepared in numbers (~10^6 cells) that are sufficient to investigate the induction of proliferation, although still too low for receptor binding studies. These cells are mainly composed by CFU-S (Table 3), but the degree of purification cannot be precisely established because of uncertainty about the coefficient of seeding. Furthermore CFU-S are a heterogeneous cell population. To account for this heterogeneity, spleen colonies were counted at day 8, when the colonies are transient and do not contain elements transplantable in secondary recipients, and at day 12. It could be argued that the sorted stem cells used in the present experiments give high numbers of day 12 CFU-S but may not be pluripotent stem cells. It should be mentioned, therefore, that the sorted cells are similarly or even more enriched for cells providing 30-day radioprotection than for day-12 CFU-S. It is difficult to make any correlation between CFU-S and cells that give rise to hematopoietic colonies in culture. However, CFU-E were undetectable and BFU-E represented only 4% of the purified cell fraction (Table 3).

Table 5. Total Cell Number and CFU-S Content in Serum-Free Cultures of Sorted Bone Marrow Cells

<table>
<thead>
<tr>
<th>Time in Culture</th>
<th>Number of Cells/Tube (x 10^6)</th>
<th>Number of CFU-S/Tube (%)</th>
<th>Number of Day-8 CFU-S/Tube (%)</th>
<th>Number of Day-12 CFU-S/Tube (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h None</td>
<td>7,091</td>
<td>100</td>
<td>368</td>
<td>787</td>
</tr>
<tr>
<td>20 h None</td>
<td>368</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 U r IL-3</td>
<td>1,295</td>
<td>18</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>400 U r IL-3</td>
<td>4,456</td>
<td>63</td>
<td>281</td>
<td>71</td>
</tr>
<tr>
<td>Ep</td>
<td>1,219</td>
<td>17</td>
<td>63</td>
<td>17</td>
</tr>
<tr>
<td>Ep + 10 U r IL-3</td>
<td>2,982</td>
<td>42</td>
<td>151</td>
<td>41</td>
</tr>
<tr>
<td>Ep + 400 U r IL-3</td>
<td>4,234</td>
<td>60</td>
<td>173</td>
<td>47</td>
</tr>
<tr>
<td>48 h None</td>
<td>410</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 U r IL-3</td>
<td>2,080</td>
<td>29</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>400 U r IL-3</td>
<td>5,988</td>
<td>84</td>
<td>191</td>
<td>52</td>
</tr>
<tr>
<td>Ep</td>
<td>680</td>
<td>10</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Ep + 10 U r IL-3</td>
<td>3,277</td>
<td>46</td>
<td>191</td>
<td>52</td>
</tr>
<tr>
<td>Ep + 400 U r IL-3</td>
<td>5,183</td>
<td>73</td>
<td>77</td>
<td>21</td>
</tr>
</tbody>
</table>

*The same experiment is presented in Table 4. Similar results were obtained in four other experiments.
The proliferation of the purified cells and the survival of day-12 and day-8 CFU-S in culture were dependent on the presence of a high concentration (400 U/mL) of rIL-3 (Tables 4 and 5). Ep plus 10 units of rIL-3 exerted effects comparable with those induced by 400 units of rIL-3. This result strongly suggests that Ep and low IL-3 concentrations cooperate to control the cell cycle of stem and progenitor cells. The time course of the cell cycle was similar to that recently described for the multilineage cell line C-633: the higher proportion of cells entered in the S + G2 + M phase between 20 and 48 hours of culture and cell proliferation was detected after 48 hours. However, the purified CFU-S did not proliferate as a synchronized cohort (Fig 7) hampering a more detailed analysis of their growth requirement in each phase of the cycle as well as of the likely transition from day-12 to day-8 CFU-S.

These results indicate that in serum-free cultures of purified stem and progenitor cells, IL-3 induces proliferation of CFU-S and multilineage colony formation in a concentration-dependent fashion. A synergistic effect between Ep and low IL-3 concentrations was observed in the induction both of CFU-S proliferation and erythroid colony formation. These data reconcile the two principal models currently available for the control of hematopoietic differentiation: the stochastic model, according to which commitment results from a stochastic event intrinsic to the stem cell, and the deterministic model, which proposes the commitment of the stem cell determined by each lineage-specific growth factor via the down modulation of the receptors for others. In fact, the data presented here indicate that at high IL-3 doses, a condition that may resemble the in vivo state of perturbed hematopoiesis, the differentiation of progenitor cells could be stochastically determined as it is independent of lineage-restricted growth factors. In contrast, at low IL-3 concentrations, which may correspond to steady-state hematopoiesis, erythroid differentiation will be determined by Ep.

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Fig 7. DNA histogram of sorted cells cultured for 20 hours in serum-deprived medium supplemented with 10 units of rIL-3 in presence (thin line) or absence (thick line) of Ep. In the cultures stimulated with Ep only, almost all the cells were in the debries area (not presented). The DNA histogram obtained in the presence of 400 units of rIL-3 was equivalent to that of rIL-3 + Ep (not presented). (The same experiment is reported in Fig 6.)
Synergism between erythropoietin and interleukin-3 in the induction of hematopoietic stem cell proliferation and erythroid burst colony formation

G Migliaccio, AR Migliaccio and JW Visser