The Production of Committed Hemopoietic Colony-Forming Cells From Multipotential Precursor Cells In Vitro

By Nicos A. Nicola and Gregory R. Johnson

Murine fetal liver cells have been fractionated by fluorescence-activated cell sorting into two fractions termed, for convenience, the pre-CFC and CFC (colony-forming cell) fractions, which differ in their relative binding of the lectin, pokeweed mitogen. The CFC fraction contained a high frequency of CFC (26%) and in liquid cultures stimulated with colony-stimulating factors generated large numbers of differentiated progeny. Consequently, residual CFC could not be detected in such cultures after day 4. In contrast, the pre-CFC fraction contained relatively few CFC (1%) but contained the majority of CFU-S and was able to generate large numbers of new CFC after 5–7 days in liquid culture. This production of CFC from precursor cells was absolutely dependent on factors present in pokeweed-mitogen-stimulated spleen cell conditioned medium (PWM-SCM) or postendotoxin serum (ES) but was not stimulated by known granulocyte or macrophage colony-stimulating factors. CFC production occurred from multipotent progenitor cells and all types of CFC were detected (excluding lymphocytes). Clonal analysis of pre-CFC showed that most of the generated CFC arose from relatively few multipotential precursors some of which could generate up to 500 CFC. The data suggested a differentiation sequence of the multipotential precursor cells in which differentiation potentials are successively restricted in the order macrophage, eosinophil, granulocyte-macrophage → granulocyte, megakaryocyte, erythroid. The frequency of CFC-generating cells in the pre-CFC fraction was significantly less (ten-fold) than that of CFU-S, suggesting that pre-CFC may be a more ancestral subset of CFU-S. The cell fractions and assay systems described should be of use in defining and purifying factors regulating early phases of hemopoietic differentiation and in defining the differentiation steps involved in the restriction of potentialities of multipotential cells.

ONE OF THE FUNDAMENTAL problems in cell biology is the mechanism by which a multipotential cell gives rise to daughter cells committed to different cell lineages and the control of this process. The hemopoietic system is ideal for studying this process because the production of multipotential stem cells of progeny committed to at least five different hemopoietic cell lineages occurs continuously throughout adult life and because the production of different types of end cells can be manipulated by externally applied stresses.1

Although factors regulating cell production from committed hemopoietic progenitor cells in vitro have been well documented2 and some have been purified1,5 factors regulating stem cell commitment (as opposed to stem cell proliferation4) are essentially unknown. Indeed, at present there is no convincing evidence that the relative frequency of stem cell commitment to a specific differentiation pathway can be altered by any factor or externally applied selective stress situation. However, in the bipotent granulocyte-macrophage cell lineage, Metcalf7 has presented evidence that the concentration of a known regulator (granulocyte-macrophage colony-stimulating factor, GM-CSF) can, at least in part, influence the direction of differentiation, and this raises the possibility that similar mechanisms might be applicable to multipotential cell differentiation. The major problems in studying stem cell commitment and any putative regulators of this process have been the unavailability of both purified stem cell populations and appropriate in vitro assay systems.

The production of committed hemopoietic progenitor cells (colony-forming cells, CFC) in liquid cultures of murine8–11 and human12–15 bone marrow has been reported, but in these studies either unfractionated or partially fractionated cells were used and the production of only one or two types of CFC was usually monitored. Moreover, in the murine system, the short time period (1–4 days) required for CFC production and the generally modest increase in total CFC number (2–10-fold) observed makes it difficult to determine whether the CFC arose from multipotential cells or early committed precursors. Continuous CFC production in long-term liquid cultures of murine16 and human17 bone marrow has also been recently reported. However, the absolute requirement for an adherent bone marrow layer for continuous cultures18 makes it difficult to assess which factors are required for CFC production and stem cell commitment.

Recently, highly purified populations of both hemopoietic stem cells (CFU-S)19,20 and their immediate committed progeny (colony-forming cells, CFC)21 have been obtained by fluorescence-activated cell sorting techniques. However, only in the rat19 and mouse21 has a good separation between CFU-S and CFC been
achieved, and of these two species, good in vitro assay systems for the different types of CFC are available only for the mouse. In the present study we have used two populations of blast cells obtained from murine fetal liver by fluorescence-activated cell sorting, one of which (CFC-fraction) is highly enriched for CFC and the other (pre-CFC fraction) depleted of CFC but only for the mouse. In the present study we have used systems for the different types of CFC are available achieved, and of these two species, fetal liver by fluorescence-activated cell sorting, one collected from CS7BL/6f/J WEHI mice 3 hr after intravenous injection of 5 µg of endotoxin (Difco, Detroit, Mich.). Mouse lung conditioned medium (MLCM) was prepared as previously described, and stage V and stage VI material were generously provided by Dr. A. W. Burgess. Pregnant mouse uterus extract (PMUE) was prepared as described by Bradley et al. and the protein precipitated between 0% and 50% saturation with ammonium sulfate used. L-cell conditioned medium (LCCM) was prepared from an L-cell line as described by Stanley and Heard. The material used in this study (partially purified by gel filtration on Ultrogel AcA44, L.K.B. Produkter, Uppsala, Sweden) was also provided by Dr. A. W. Burgess. All stimuli were pretitrated for colony-stimulating activity to ensure no high dose inhibition was present and used at a final concentration 5–10 times that needed for maximal colony formation.

**MATERIALS AND METHODS**

**Fractionation of Fetal Liver Cells Into Pre-CFC and CFC Fractions**

Full details of cell preparation, labeling, and sorting by fluorescence-activated cell sorting have been published previously. Briefly, 13–14-day murine fetal livers (CBA/CaH WEHI strain) were dissected, a single cell suspension made, and cells of density less than 1.077 g cc⁻¹ collected. The light density cells were labeled with fluorescein isothiocyanate-conjugated pokeweed mitogen and then with a rabbit antineutrophil serum, followed by rhodamine-conjugated sheep anti-rabbit immunoglobulin antibody. The cells were analyzed and sorted on a Becton-Dickinson FACS II instrument, measuring independently red and green fluorescence and low angle light scatter intensity. Both cell fractions were selected for negative red fluorescence and high intensity low angle scatter characteristics. The pre-CFC fraction was selected for relatively low green fluorescence intensity (channels 40–90) and the CFC fraction for high green fluorescence intensity (channels 130–250). (Channel numbers refer to Fig. 5 of Nicola et al.) Both fractions contained essentially pure populations of undifferentiated blast cells with the functional characteristics shown in Table 1.

**Sources of Stimulating Factors**

Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared as described previously. The material used in the present study was dialyzed against distilled water and concentrated fivefold in phosphate-buffered (0.2M, pH 7.3) saline (0.15M) (PBS) using a hollow fiber apparatus (Amicon DC2, Amicon, Boston, Mass.) Protein precipitated between 0% and 50% saturation with ammonium sulfate used. L-cell conditioned medium (LCCM) was prepared from an L-cell line as described, and stage V and stage VI material were generously provided by Dr. A. W. Burgess. All stimuli were pretitrated for colony-stimulating activity to ensure no high dose inhibition was present and used at a final concentration 5–10 times that needed for maximal colony formation.

**Liquid Cultures of Pre-CFC and CFC Fractions**

For most experiments, 50,000 cells of either fraction were added to individual wells in 24-well Costar tissue culture clusters (Costar, Cambridge, Mass.). The wells had been precultured with 0.5 ml of double strength Dulbecco’s Modified Eagle’s medium (DME) containing 40% (v/v) fetal calf serum for 3–5 hr in a humidified 10% CO₂ incubator to detoxify the wells. The medium was then replaced with 0.5 ml RPMI 1640 medium, 20% (v/v) fetal calf serum, the cells, and 10% (v/v) stimulus. In some experiments the number of cells added was 25,000 or 100,000 to check for linearity of the responses. The trays were then incubated for 7–8 days in a fully humidified incubator with 10% CO₂ in air at 37°C. At daily intervals, small aliquots (25–100 µl) of nonadherent cells were removed for viable cell counting, differential cell counts, and assay in agar cultures.

**Semisolid Cultures**

Counts of viable cells were performed using eosin exclusion, and agar cultures for CFC were performed as described previously. For secondary agar cultures, 200 cells from CFC liquid cultures or 2000 cells from pre-CFC liquid cultures were plated per 1 ml agar-medium. For all agar cultures 5–10 times maximal concentrations of PWM-SCM were used to optimize detection of all possible CFC. For primary semisolid cultures of the CFC and pre-CFC fractions, 200 or 2000 cells, respectively, were cultured either in agar-medium or, for recloning experiments, in methylcellulose. The latter cultures (1 ml) contained 0.9% methylcellulose, 20% fetal calf serum, 10% PWM-SCM, and Iscove’s modified Dulbecco’s medium. For recloning analyses, individual sequential colonies were removed from 7-day cultures with a finely drawn Pasteur pipette, resuspended in 2.5 ml of warm agar-medium, and 2 ml of this mixture cultured in duplicate Petri dishes (35 mm). The CFC content of primary methylcellulose colonies was determined from the average number of agar colonies (after a further 7 days) in each of these secondary cultures multiplied by 2.5.

**Table 1. Functional Properties of the CFC and Pre-CFC Cell Fractions**

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>CFC Frequency</th>
<th>CFC-S Frequency</th>
<th>CFU-E Frequency</th>
<th>Primary Colony Type (CFC) (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFC</td>
<td>26 ± 6</td>
<td>0.9 ± 0.2</td>
<td>0</td>
<td>G 13</td>
</tr>
<tr>
<td>Pre-CFC</td>
<td>1 ± 0.3</td>
<td>2.6 ± 0.7</td>
<td>7 ± 5</td>
<td>15 20</td>
</tr>
</tbody>
</table>

*Primary colonies stimulated in agar cultures with PWM-SCM for 7 days.
†Measured at day 8 as spleen nodules (surface) in irradiated and transplanted mice, assuming a seeding factor of 0.05.
‡Colony morphology of stained cultures of primary cultures in agar cultures stimulated with PWM-SCM. G, granulocyte (neutrophil); GM, granulocyte-macrophage; M, macrophage, B, blast cells; E, erythroid; MEG, megakaryocyte; EO, eosinophil; MIX, mixed erythroid.

Average results of 5–10 separate experiments (± standard deviation) except for colony types, which are the average from 3 experiments.
**CFU-S Assays**

CFU-S content of cell fractions was determined by the number of spleen colonies observed 8 days after lethal irradiation (850 rads, x-irradiation) and transplantation into syngeneic mice. The number of colony-forming cells (CFC-S) was calculated assuming a seeding factor for fetal cells into adult of 0.05, as described previously.2°

**Typing of Colonies**

The types of CFC generated in liquid cultures were determined by staining the cells in colonies generated from the CFC in 7-day agar cultures. The entire agar culture dish was flooded with 5% glutaraldehyde (v/v) in PBS for 3 min, followed by absolute methanol for 4 hr. The agar gel was then rimmed with a spatula and allowed to float on water. The gel was removed to a large glass slide, covered with a wet Whatman no. 541 filter, and allowed to dry at room temperature. After removal of the filter paper, the thin film of gel containing flattened colonies was stained. Duplicate gels were stained either for acetylcholinesterase or with Luxol Fast Blue to detect eosinophil granules and counterstained with Meyer’s hematoxylin. The acetylcholinesterase stain for megakaryocytes used in this way has been described previously.25 The Luxol Fast Blue stain was used as described previously for individual colonies and the procedure for hematoxylin staining has been described previously.21 Erythroid cells and granulocytes and macrophages were easy to classify on the basis of hematoxylin staining alone. In a few cases, the percentages of these colony types were confirmed by benzidine staining for erythroid colonies and nonspecific esterase staining for macrophages.

**RESULTS**

We have recently shown that murine fetal liver hemopoietic progenitor cells can be separated into different functional classes of blast cells on the basis of their quantitative binding of the lectin pokeweed mitogen using fluorescence-activated cell sorting. In particular, CFU-S showed a bimodal distribution, with about 25% of CFU-S occurring in the highly fluorescent cell fraction that was greatly enriched (to about 26%) for in vitro colony-forming cells (CFC), while about 75% of CFU-S occurred in a much less fluorescent cell fraction, which contained CFC at a frequency of only 1%. CFU-E showed even less fluorescence than the majority of CFU-S and contaminated only the latter fraction (to a level of 7%). For convenience, the fraction of cells most highly enriched for CFC will be termed the CFC fraction and that most highly enriched for CFU-S will be termed the pre-CFC fraction. Both fractions of cells were essentially morphologically pure populations of undifferentiated blast cells. The content of CFC, CFU-S, CFU-E, and the types of primary colonies generated by each fraction of cells in agar cultures stimulated by pokeweed-mitogen-stimulated spleen cell conditioned medium (PWM-SCM) are shown in Table 1. Of particular interest was the observation that most of the erythroid and essentially all of the mixed colonies detectable in 7-day primary cultures were generated by cells from the CFC fraction, while the pre-CFC fraction generated a higher frequency of colonies of undifferentiated blast cells.

When the two cell fractions were seeded in liquid cultures at 75,000 cells/ml and stimulated with optimal levels of PWM-SCM, the results shown in Fig. 1 were obtained. As described previously, in liquid cultures of the CFC fraction, cell numbers increased dramatically (30-40-fold) during the first 4 days of culture, while CFC numbers (as determined from secondary agar cultures of the liquid-cultured cells at daily time points) declined to zero between days 3 and 5. In contrast, liquid cultures of the pre-CFC fraction showed little increase in cell numbers during the first 5 days of culture, but CFC numbers increased about 25-fold between days 3 and 5 of liquid culture. After the peak in CFC numbers at day 5, the total cell...
production in liquid cultures of the pre-CFC fraction increased to over 10-fold by day 8. The transient increase in cell numbers (2-fold) seen at day 2 of pre-CFC liquid cultures appeared to be due to proliferation of contaminating CFU-E as described previously, a conclusion supported by smears of the cultured cells at this time point that showed about 50% maturing and mature erythroid cells.

The effects of different stimuli on the production of CFC from the pre-CFC fraction in liquid culture are shown in Fig. 2. Significant production of CFC was absolutely dependent on factors present in PWM-SCM or postendotoxin serum (ES). Neither crude nor highly purified (stage VI) GM-CSF from mouse lung conditioned medium (MLCM) was able to stimulate production of CFC despite the ability of GM-CSF to stimulate the early proliferation of many different kinds of CFC, including mixed CFC. Addition of macrophage-specific CSFs (L-cell conditioned medium, pregnant mouse uterus extract), normal mouse serum, or normal saline also failed to stimulate CFC production in liquid cultures of the pre-CFC fraction (data not shown). Despite the fact that both the PWM-SCM and ES were used at concentrations that were at least five-fold higher than that required for maximal stimulation of CFC proliferation and did not show high dose inhibition, the effects of PWM-SCM and ES on CFC production were additive (Fig. 2). Similarly, cell production in liquid cultures of the pre-CFC fraction was increased between days 5 and 7 when the combined stimuli were used (Fig. 2).

The effect of fetal calf serum (FCS) on CFC production from the pre-CFC fraction was also assessed. With no added FCS, the number of CFC produced in PWM-SCM-stimulated liquid cultures was similar to that produced in FCS-containing cultures, although both CFC production and cell production appeared to be delayed by about 1 day. Although there was some human serum present in these cultures (present in the added PWM-SCM), this was calculated to be about 4% (v/v, final concentration). The same amount of PWM-SCM added to liquid cultures of the CFC fraction in the absence of FCS resulted in a marked reduction (by 80%) of cell production compared to FCS-containing cultures (data not shown).

The time course of production of different types of CFC in PWM-SCM-stimulated liquid cultures of the pre-CFC fraction is shown in Figs. 3 and 4. It can be seen that all the different kinds of CFC that can be detected in PWM-SCM agar cultures were produced in the liquid cultures, including granulocyte (G), macrophage (M), granulocyte-macrophage (GM), eosinophil (EO), megakaryocyte (MEG), and mixed erythroid (MIX) CFC. However, as seen in Fig. 3, there was a distinct change in the proportion of different types of CFC produced at different time points in the liquid cultures. Nearly 80% of the CFC produced by day 4 of liquid culture

![Fig. 2. Production of CFC and viable cells in liquid cultures of the pre-CFC fraction (50,000 cells seeded per culture). Cultures were stimulated with PWM-SCM (O-O), ES (---), PWM-SCM + ES (O-), or stage VI MLCM (Δ-). Typical results of 2-6 such experiments.](image-url)
generated pure macrophage colonies, but this proportion decreased nearly to zero by day 8 of liquid culture. In contrast, the proportion of erythroid and granulocyte CFC increased during this same time period (E-CFC from 9% to 63%, G-CFC from 0% to 22%). Towards the end of the culture period (day 8), only E-CFC, G-CFC, MIX-CFC, and MEG-CFC were detected. E-O-CFC were detected only on days 5–6 and GM-CFC were detected on days 4–7. Since total CFC production levels varied markedly during the culture period (see Figs. 1 and 2), the total production of each type of CFC can be plotted in Fig. 4 by multiplying the total frequency of each CFC type at each time point by the total number of CFC measured at each time point. The data were calculated by multiplying the frequency of each CFC type at each time point by the total number of CFC measured at each time point.

GM-CFC and MIX-CFC production also peaked at day 5, although the shape of both of these curves demonstrated a shift of nearly 1 day to longer time points compared to M-CFC production. MEG-CFC, E-CFC, and G-CFC production were all clearly shifted to longer time points, peaking between days 5 and 7.

The differentiation potentials of the mixed-erythroid CFC detected between days 4 and 8 of liquid culture...
cultures of the pre-CFC fraction were determined by typing the cells subsequently formed in agar colonies by MIX-CFC. The results are shown in Table 2. All mixed colonies observed contained erythroid cells regardless of the liquid culture time point at which the cells were seeded in agar cultures. Only at early liquid culture time points (days 4–6) were MIX-CFC with potentials for more than two cell lineages detected. Because of the staining techniques used, eosinophils and megakaryocytes could not be simultaneously stained in the same colony, but it appears that the eosinophil differentiation potential of MIX-CFC was lost well before megakaryocyte differentiation potential, and the granulocyte-macrophage differentiation potential was lost at an intermediate time point. The most striking observations were the increased restriction of MIX-CFC to erythroid and megakaryocyte differentiation potentials from day 6 to 8 and the absence of MIX-CFC restricted to other combinations of differentiation potentials (e.g., EO, GM-E, MEG-GM included in “other” category of Table 2).

These data suggest that CFC production in liquid cultures of the pre-CFC fraction occurs from multipotential cells and might suggest a differentiation sequence from multipotential cells to committed CFC. However, it is not possible from these data to determine what proportion of newly produced CFC arose from multipotential cells or from precommitted cells. In order to answer this question, clonal analysis of CFC production is required and was performed by seeding the pre-CFC fraction directly into methylcellulose with PWM-SCM and FCS. After 7 days, sequential individual colonies and clusters were picked off, resuspended, and recultured in agar cultures stimulated with PWM-SCM to detect the number of CFC produced in the original colony. The results of this study are shown in Fig. 5 and Tables 3 and 4.

Figure 5 shows the range of CFC numbers in primary methylcellulose colonies formed by cells from either the CFC fraction or the pre-CFC fraction. Only 4/142 primary colonies generated by cells in the CFC fraction were positive for CFC, and these contained from 7 to 50 CFC. In contrast, 54/273 primary colonies generated by cells in the pre-CFC fraction were positive for CFC, and these contained from 2 to

<table>
<thead>
<tr>
<th>Day of Liquid Culture</th>
<th>Number of Mixed Colonies Observed Containing*</th>
<th>EO + GM + E</th>
<th>MEG + GM + E</th>
<th>MEG + E</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>5</td>
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<td>7</td>
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<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

*Pre-CFC fraction cells were seeded at 50,000 per liquid culture stimulated with PWM-SCM. At the indicated liquid culture time points, cells were removed and plated in agar cultures also stimulated with PWM-SCM (2000 cells/plate). Seven days later the agar cultures were stained, and those colonies containing more than one lineage of hemopoietic cells (excluding granulocyte-macrophage colonies) enumerated as well as their cell type contents. However, since a constant number of plates was not examined for each time point, only the relative content of different types of MIX-CFC at each time point is meaningful. Abbreviations are as for Table 1. EO + GM + E and MEG + GM + E are not mutually exclusive categories, since individual plates could not be stained simultaneously for EO and MEG. No other mixed colony types than those listed were detected ("other" category).
Clusters were 3-49 cells and colonies were more than 50 cells (as for further cultures stimulated with PWM-SCM by cells colonies generated gross morphology and appeared white. Colonies that replated.)

Individual primary colonies were then removed and replated in agar cultures supplemented with PWM-SCM for 7 days. Colonies that generated all the primary colonies tested. (Every colony and cells generated.

Table 3 shows that the CFC content of primary colonies generated by cells in the pre-CFC fraction closely approximated the expected number of CFC that would be generated in equivalent liquid cultures at day 7, indicating that the cells that give rise to CFC are detectable as primary colonies in methylcellulose rather than clusters or single cells. In parallel experiments, the number of CFC in the entire methylcellulose plate was compared to that found in individual colonies. It was found that the sum of CFC in individual colonies was essentially equal to that found when the entire plate contents of duplicate cultures was recultured.

Table 4 shows that the majority of CFC were generated from multipotent cells. In cultures of the pre-CFC fraction, 17 of the 273 primary colonies contained 88% of all the generated CFC. Of these 17 colonies, 13 gave rise to erythroid as well as nonerythroid (predominantly granulocyte-macrophage) CFC, indicating the multipotentiality of the originating cells; 3 gave rise to only erythroid CFC and 1 gave rise to only GM-CFC. From these data (Tables 3 and 4) it could be calculated that 75% of all CFC produced by day 7 of culture had probably been produced from multipotent cells. It can also be calculated from Table 3 that the frequency of cells in the pre-CFC fraction that generate colonies that contain CFC is only 0.2% compared to 0.8% in the CFC fraction. This is despite the fact that, on a per cell basis, the pre-CFC fraction generates many more CFC at days 5-7 than does the CFC fraction (Fig. 1) and indicates that cells in the pre-CFC fraction have a much greater capacity to generate CFC than those in the CFC fraction, although the latter are more numerous. In the CFC fraction, the frequency of cells that generate CFC is about the same as the frequency of CFU-S, but in the pre-CFC fraction, the frequency of such cells is less than a tenth of the frequency of CFU-S (Table 3).

**DISCUSSION**

The results presented here have shown that cells that generate CFC (pre-CFC) can be separated from CFC on the basis of their relative binding of the lectin, pokeweed mitogen. Previous studies on both murine and human bone marrow have shown that cells generating CFC can be partially separated from CFC on the basis of sedimentation velocity, since the former are smaller cells. The present separation technique, however, has the advantage that the separation appears to be more complete and the two fractions can be obtained as highly enriched populations of blast cells free of mature cells that may modulate hemopoiesis.

CFC generation in liquid cultures of the pre-CFC fraction differed from that in liquid cultures of the CFC fraction. In the CFC fraction, CFC numbers

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**Table 3. Clonal Analysis of CFC Production From Precursor Cells in the CFC and Pre-CFC Cell Fractions at Day 7 of Primary Culture**

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Pre-CFC</th>
<th>CFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of primary colonies replated</td>
<td>273</td>
<td>142</td>
</tr>
<tr>
<td>No. giving 0 secondary colonies (%)</td>
<td>220 (80%)</td>
<td>138 (97%)</td>
</tr>
<tr>
<td>No. giving &gt;25 secondary clusters (%)</td>
<td>128 (47%)</td>
<td>10 (7%)</td>
</tr>
<tr>
<td>No. giving 2-14 secondary colonies (total secondary)</td>
<td>29 (130)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>No. giving 15-38 secondary colonies (total secondary)</td>
<td>8 (190)</td>
<td>2 (38)</td>
</tr>
<tr>
<td>No. giving 40-100 secondary colonies (total secondary)</td>
<td>7 (481)</td>
<td>1 (51)</td>
</tr>
<tr>
<td>No. giving 110-200 secondary colonies (total secondary)</td>
<td>7 (801)</td>
<td>0</td>
</tr>
<tr>
<td>No. giving 201-550 secondary colonies (total secondary)</td>
<td>3 (1048)</td>
<td>0</td>
</tr>
<tr>
<td>No. of primary clusters replated</td>
<td>68</td>
<td>54</td>
</tr>
<tr>
<td>No. giving 0 secondary colonies</td>
<td>68</td>
<td>54</td>
</tr>
<tr>
<td>No. giving 1-10 secondary clusters</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total secondary colonies generated</td>
<td>2,650</td>
<td>97</td>
</tr>
<tr>
<td>Expected total secondary (liquid culture)†</td>
<td>3,000</td>
<td>0</td>
</tr>
<tr>
<td>Percent primary colonies giving secondary colonies</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Percent cells in fraction giving secondary colonies (pre-CFC)</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Percent cells in fraction that are CFU-S</td>
<td>2.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

†Calculated from the number of CFC generated in liquid cultures of 50,000 cells divided by 50,000 and multiplied by the total number of cells that generated all the primary colonies tested. (Every colony and cluster from primary methylcellulose cultures was removed and replated.)
increased by a small factor (about twofold), occurred only for the first 3 days, and this was observed with any source of GM- or M-CSF. This apparent increase in CFC numbers is most probably the result of cell divisions of CFC with a relatively high proliferative capacity (say the ability to generate 1000 cells), so that after 2-3 cell divisions, each progeny cell can still generate enough differentiated cells (50 cells) to be enumerated as a CFC. The general decrease in colony size generated by CFC with time in such liquid cultures is consistent with this interpretation. Several other reports that have demonstrated CFC generation in liquid cultures of unfractionated or partially fractionated cells have generally used early time points to measure CFC production and measured the production of only one or two types of CFC so that it is difficult to assess whether CFC production occurred from multipotential cells or from CFC as described above. An additional problem is that some of the CSF sources used were M-CSFs, which may detect relatively mature monocytes and macrophages as CFC.

In contrast, CFC production in liquid cultures of the pre-CFC fraction occurred after an apparent delay of 3 days in liquid culture, the increase in CFC numbers was large (25-40-fold), and was absolutely dependent on factors present in PWM-SCM and/or ES. It could be distinguished from CFC production by cells from the CFC fraction, in that other sources of GM- or M-CSF (some of which have been used in the other studies mentioned above) were unable to stimulate CFC production from pre-CFC cells and by the lesser dependence on possible accessory factors present in fetal calf serum of CFC production compared to CFC proliferation. The ability of ES to stimulate CFC production from pre-CFC cells in vitro is of interest in view of the observation that transfer of ES to otherwise untreated mice induces a dramatic rise in total numbers of CFC of all types in the spleen in vivo. Studies in long-term bone marrow cultures, liquid cultures, and agar cultures have suggested that adherent bone marrow cells can sustain CFC production in the absence of detectable levels of GM- or M-CSF. Although we have reported previously that pure GM-CSF can stimulate early proliferative events in a proportion of multipotential cells that form 7-day mixed colonies, CFC were rare in such colonies, and in cell separation experiments, the majority of mixed CFC could be separated from the pre-CFC fraction used in these studies. However, since the pre-CFC cells were multipotential and generated nearly all types of CFC by day 7 of liquid culture they would be expected to form mixed colonies in primary agar cultures only after 14 days of culture. Preliminary studies have, in fact, shown that in spleen, a much higher proportion of day-14 mixed colonies contain CFC than do day-7 mixed colonies.

It should be emphasized that the culture system for production of CFC from the pre-CFC fraction is not necessarily optimized. Although either PWM-SCM or ES could stimulate CFC production, their effect was additive. This could indicate either that stimuli in the two sources act on different subpopulations of pre-CFC cells or that, although the stimuli were supramaximal for CFC proliferation, they were not maximal for pre-CFC proliferation. Preliminary data suggest that the latter possibility may be correct, since CFC production did not reach plateau levels when the PWM-SCM and ES were titrated up to the concentrations used in the present study. Moreover, no significant difference was observed in the relative proportions of different types of CFC produced in liquid cultures of the pre-CFC fraction stimulated with one or the other stimulus. Although it seems clear that neither the GM-CSF nor the M-CSF in PWM-SCM and ES is responsible for CFC production, their presence at supramaximal levels in these sources may reduce the number of CFC detected in the liquid cultures by providing a strong differentiative stimulus. It will be of interest to determine if the stimulus for production of CFC can be separated from the known CSFs present in PWM-SCM and ES or if other sources (such as bone marrow conditioned medium) can be found that have stronger pre-CFC stimulation with less CSF. The reason for the 3-day delay before CFC production begins in liquid cultures of the pre-CFC fraction is at present unknown, but the possibility that it is due to suboptimal culture conditions is being investigated.

It is clear from the clonal analysis that the majority of

<table>
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<th>Table 4. Clonal Analysis of Multipotentiality of Pre-CFC Cells*</th>
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<tr>
<td>17 primary colonies in pre-CFC fraction generated 88% of all CFC produced</td>
</tr>
<tr>
<td>7 primary colonies contained 40-100 CFC each 4 contained erythroid + nonerythroid CFC</td>
</tr>
<tr>
<td>7 primary colonies contained 110-200 CFC each 6 contained erythroid + nonerythroid CFC</td>
</tr>
<tr>
<td>3 primary colonies contained 201-550 CFC each 3 contained erythroid + nonerythroid CFC</td>
</tr>
<tr>
<td>75% of all CFC detected were generated by multipotential pre-CFC (with erythroid + nonerythroid potentials)</td>
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*This analysis is from the same data set as for Table 3. Secondary colonies (CFC produced in primary colonies) were detected in PWM-SCM-stimulated cultures and colonies typed by gross morphology and color either as erythroid or nonerythroid.
of CFC produced in these cultures is generated from multipotential cells, since CFC for all the major hematopoietic lineages (with the possible exception of lymphocytes) were detected. However, the relationship of the pre-CFC cell to CFU-S and MIX-CFC is unclear. Since the pre-CFC fraction is well separated from about 25% of the CFU-S and the majority of day-7 MIX-CFC on the basis of pokeweed mitogen binding,21 the three classes of cells cannot be completely equated. The majority of CFU-S occur in the pre-CFC fraction, but clonal analysis indicated that cells generating CFC in vitro were at least tenfold less frequent than CFU-S in this fraction. Furthermore, preliminary data (Johnson and Nicola) have shown that only a small proportion of CFU-S in this fraction generated spleen colonies that contained CFC and secondary CFU-S. Other experiments32 have suggested the existence of two types of CFU-S, the multipotential subset forming day-14 spleen colonies and the restricted subset forming day-7 spleen colonies. This suggests that pre-CFC may be a primitive subset of CFU-S and, as mentioned earlier, a subset of day-14 MIX-CFC. Indeed, some CFU-S are also generated in liquid cultures of the pre-CFC fraction (Johnson and Nicola, unpublished). It has been suggested33 that hematopoietic stem cells form a hierarchy of cells of decreasing self-renewal and increasing differentiation capacity. CFU-S, MIX-CFC, and pre-CFC assays may each measure overlapping but different classes of these cells, each class with its own heterogeneity.

Clonal analysis also indicated that the majority of CFC produced in the liquid cultures originated from a relatively small number of multipotential cells with a high proliferative potential (some generating up to 550 CFC). The change in the types of CFC produced at different time points in the liquid cultures might therefore indicate a predetermined differentiation sequence of primitive multipotential cells into committed precursor cells (CFC). Both the change in CFC production in liquid cultures and especially the successive restriction in differentiation potentials of MIX-CFC with increasing time in liquid culture suggest the differentiation sequence shown in Fig. 6.

In this sequence, M-CFC are generated first before GM-CFC. Although it may be thought that M-CFC would be generated by further restriction of bipotent GM-CFC, the data do not support this view, since large numbers of M-CFC were detected in the liquid cultures before the appearance of relatively low numbers of GM-CFC. M-CFC of high proliferative potential (HPP-CFC) have been observed by Bradley and Hodgson34 in regenerating murine bone marrow after treatment with 5-fluorouracil. Their observation35 that such CFC correlated better with the repopulating ability of bone marrow samples than did CFU-S or other types of CFC might be consistent with our observation that M-CFC are closer in generation time to multipotential precursors of CFC than other CFC and possibly even some CFU-S.

Moreover, HPP-CFC require dual stimulation by PWM-SCM and M-CSF for detection as very large colonies and such colonies usually contain many CFC.34-36 This may suggest that HPP colonies represent proliferation from a pre-CFC, with accompanying expansion of the M-CFC thus generated.

At the other end of the sequence, the data strongly suggest that MEG-CFC and E-CFC are the last to be generated from pre-CFC. This would explain why erythroid cells are nearly always seen in mixed colonies,37 and our prediction would be that megakaryocytes (which need to be appropriately stained to be detected) would also be obligatory components of all mixed colonies. In fact, a sequence similar to that in Fig. 6 has been previously suggested from an analysis of data on the types of cells that occur in mixed colonies.37 The intermediate differentiation events are less clear, but commitment to EO-CFC probably precedes commitment to GM-CFC on the basis of the
data in Table 2. It is assumed that many G-CFC arise from GM-CFC, because G-CFC were only detected in the liquid cultures at late time points (equivalent to that for E-CFC) after GM-CFC production. If they arose independently from MIX-CFC, as do E-CFC, we should have detected mixed colonies containing, for example, only erythroid and granulocyte cells.

Although the tentative differentiation sequence in Fig. 6 is consistent with all the data presented in this article, it is dependent on the assumption that most of the CFC generated in the liquid cultures arose from essentially a single type of multipotent pre-CFC. It would be invalid if, for example, M-CFC arose from a relatively mature pre-M-CFC (hence taking only 3 days to generate M-CFC), while E-CFC arose from a relatively immature pre-CFC (taking 6–7 days to generate E-CFC). Clonal analysis suggested that this was not the case, but this possibility cannot be excluded. Current experiments on the kinetic analysis of production of different kinds of CFC from single clones of pre-CFC cells should help to resolve this question. It should also be noted that all the studies in this report were done with fractions of fetal hemopoietic progenitor cells. Since there is evidence that fetal stem cells differ from adult stem cells in some physical characteristics (size and density) as well as some functional characteristics (self-renewal and hemopoietic repopulating ability), it remains to be determined if the above properties of pre-CFC cells are common to those for the adult counterpart cells.

Another question not addressed in the present report is that of the self-renewal capacity of pre-CFC as well as the possible production of CFU-S from pre-CFC. The cell fractions and assay systems described in this report should allow approaches to these questions as well as allowing attempts to purify the appropriate regulatory molecules.

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The production of committed hemopoietic colony-forming cells from multipotential precursor cells in vitro

NA Nicola and GR Johnson