Limiting-Dilution Analysis of the Effects of Colony-Stimulating Factors, Phytohemagglutinin, and Hydrocortisone on Hematopoietic Progenitor Cell Growth

By Yoichi Takaue, Christopher L. Reading, Aaron J. Roome, Karel A. Dicke, Sharon Tindle, Meena Chandran, and Barbara Devaraj

The effects of colony-stimulating factors (CSFs), phytohemagglutinin (PHA), and hydrocortisone on the growth of human bone marrow hematopoietic progenitor cells (granulocyte-macrophage; GM) were analyzed in a limiting-dilution assay (LDA). Both low-density bone marrow cells separated by discontinuous Percoll gradients and a T cell–depleted and progenitor-enriched cell fraction obtained by the combination of counterflow elutriation centrifugation and Percoll gradients were examined in LDA. GCT (monocyteoid cell line–conditioned medium containing GM-CSF), human placenta–conditioned medium, bladder carcinoma cell line BS57–conditioned medium (containing GM- and G-CSF), and recombinant CSF (G-CSF) directly induced proliferation of progenitors with single-hit kinetics. In some instances, however, PHA-stimulated lymphocyte-conditioned medium (containing G- and GM-CSF) showed deviation from single-hit kinetics, which demonstrated the presence of factor(s) suppressive to progenitor growth. In a T cell–depleted, progenitor-enriched fraction, PHA alone was found to suppress progenitor growth at a level as low as 100 ng/mL. The addition of hydrocortisone (10^-8 mol/L) increased the progenitor frequency but suppressed progenitor growth at 10^-4 mol/L. LDA appears to be a valuable method for exploring mechanisms of factors regulating hematopoietic cell growth.

G R A N U L O C Y T E - M A C R O P H A G E (GM) colony–stimulating factors (CSFs) are glycoproteins that regulate growth and differentiation of GM progenitors. Agar and methylcellulose colony assays have been used to evaluate the effects of CSF, to monitor purification of committed hematopoietic progenitors, and to represent a correlate of hematopoietic restoration potential of bone marrow (BM) aspirates.

Colony assays in semisolid media have aided the understanding of hematopoiesis, but they have limitations. The different preparations of CSFs used and the criteria used to define clonal growth make colony results from various laboratories difficult to compare. Agar or methylcellulose may disrupt cellular and extracellular matrix interactions that play a role in hematopoietic proliferation and differentiation. These assays are not linear except at relatively high cell concentrations, and under these conditions, the number of colonies obtained may be influenced by helper or suppressor accessory cell functions. To conclude that a factor has a direct effect on progenitors, it should be demonstrated that the observed colony growth is based on single-hit kinetics (ie, the factor is affecting only one cell type and not operating via an accessory cell). In these culture systems, assessment of the effect of CSFs on progenitor growth requires purified progenitors. However, human hematopoietic progenitor purification is time and labor intensive and has met with only limited success.

Limiting-dilution analysis has been used to enumerate murine hematopoietic progenitors, and recently, we have developed a quantitative assay of human BM GM progenitors by limiting-dilution assay (LDA). This method was shown to detect approximately three times more total progenitors than methylcellulose colony assays. LDA offers the potential for examining mechanisms of factors regulating progenitor growth in vitro and, hence, is an ideal method for resolving several of the aforementioned problems. Using this method, we have evaluated the stimulatory activities of CSFs on progenitor growth by using low-density BM cell fractions from Percoll gradients. The effects of phytohemagglutinin (PHA) and hydrocortisone (HC) on progenitor growth were also analyzed with a T cell–depleted and progenitor-enriched fraction that was obtained by the combination of counterflow elutriation centrifugation (elutriation) and Percoll gradient separation.

We have found that a human giant cell tumor line (GCT), human placenta–conditioned medium (HPMC), bladder carcinoma–conditioned medium (BCM), and recombinant CSF can stimulate the growth of hematopoietic progenitors independent of the effects of accessory cells. PHA suppressed CSF-stimulated progenitor growth, and HC increased the progenitor frequency at 10^-4 mol/L. This analysis allows evaluation of various agents that affect hematopoietic cell growth in vitro.

MATERIALS AND METHODS

**BM Cell Fractionation**

BM aspirates were fractionated to enrich for mononuclear cells as previously described. Without this enrichment, it was difficult to detect colonies because of the greater number of nonproliferating cells. Briefly, BM was aspirated from normal donors after obtaining informed consent. Cells were centrifuged at 600 g for 25 minutes, and buffy coat cells (BMBC) were collected. A stock solution of 100% Percoll was prepared by diluting Percoll (Sigma Chemical Co, St Louis) with 1/10 volume of 10x phosphate-buffered saline (PBS). The 100% solution was then diluted with Dulbecco's PBS (GIBCO, Grand Island, NY) to obtain 60% and 40% Percoll.

From the Departments of Pediatrics, Tumor Biology, and Hematology, The University of Texas M.D. Anderson Hospital and Tumor Institute, Houston.

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Address reprint requests to Christopher L. Reading, PhD, Monoclonal Antibody Laboratory, Box 179, M.D. Anderson Hospital and Tumor Institute, 6723 Bertner Ave, Houston, TX 77025.

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solutions. BMBC were layered over a 8-mL gradient consisting of 4-mL layers of 60% and 40% Percoll (Sigma) in 15-mL polystyrene tubes (Becton Dickinson, Oxnard, CA). These were centrifuged for 20 minutes at 750 g. Cells in the 40/60 Percoll interphase were aspirated and washed twice with Hank’s balanced salt solution supplemented with 1% fetal bovine serum (FBS). The 40/60 Percoll fraction is depleted of erythrocytes, granulocytes, platelets, and nucleated red cells and enriched in immature blasts. Cells thus obtained (Fr 40/60) were used to study the effect of CSFs on progenitor growth.

LDA of Progenitors

LDA was performed as previously described. Briefly, BM cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.6 × 10^{-5} mol/L NaHCO₃, 1.8 × 10^{-5} mol/L HEPES, 3 × 10^{-4} mol/L hypoxanthine, 3 × 10^{-8} mol/L thymidine, 10^{-5} mol/L pyruvate, 5 × 10^{-5} mol/L 2-mercaptoethanol, 10^{-4} mol/L minimal essential medium (MEM) nonessential amino acids (GIBCO), 15% FBS, and CSF at various concentrations. For the study of CSFs, 10^{-4} mol/L HC sodium succinate (Ekins-Sinn, Inc., Cherry Hill, NJ) was added to the medium to suppress the growth of residual T cell clones. The cell suspension was mixed by pipetting and placed in 60-well microplates (Flow Laboratories, McLean, VA) at seven to nine cell densities (10 to 400 cells/10 μL/well).

Each dilution consisted of one or two plates (60 to 120 wells). The plates were then incubated in a humidified atmosphere (37°C, 5% CO₂ in air), and the medium was replaced every other day. After 10 days the number of cells in the largest cluster of blasts in each well was counted by using an inverted microscope. Control wells were incubated without CSFs.

Statistical Analysis

Linear regression analysis was applied to the limiting dilution data to calculate the best slope. The resulting straight line allowed calculation of the progenitor frequency. Confidence intervals for the slope were calculated by using a form of Student’s t test. Conformance of the data to linearity was tested by using a chi-square analysis; a probability P > .05 indicated single-hit kinetics, thus demonstrating the activity of a single limiting cell type.

Morphological Evaluation

The cells were examined morphologically by using Wright's stained cytocentrifuge preparations (Shandon Products, Astmoor, England). To minimize distortions, slides were also made by unit gravity sedimentation. This was especially useful in examination of immature blasts. The slides were also analyzed for nonspecific esterase, naphthol-AS-D-chloroacetate esterase, and myeloperoxidase (Sigma) by following the manufacturer's instructions.

CSFs

Five types of CSFs were evaluated at various concentrations. HPCM, prepared by the method of Burgess et al., was tested at 1% to 20%. PHA-stimulated leukocyte-conditioned medium (PHA-LCM) was prepared by using the method of Iscove et al. with minor modifications. Normal donor peripheral mononuclear cells were cultured at 2 × 10⁶ cells/mL in DMEM with 7.5% FBS and 7.5% human AB serum in the presence of PHA (10 μg/mL, Sigma) at 37°C, 5% CO₂ for four days. The medium was harvested, filtered, and tested at 1% to 10%, with and without 1 U/mL erythropoietin (Epo; Connaught, Toronto, Ontario, Canada). A single lot of the culture medium of GCT (GIBCO) was used at 1% to 20%. Conditioned medium of human bladder carcinoma cell line 5637 (kindly provided by Dr Fogh, Memorial Sloan-Kettering Cancer Center) was tested at 0.5% to 10%. The 5637 cells were cultured in RPMI 1640 supplemented with 10% FBS, and the medium was harvested every three or four days, filtered, and stored at 4°C. Recombinant CSF, provided by Dr Souza, Amgen, Inc (Thousand Oaks, CA), was tested at 10 to 500 U/mL.

Effects of PHA and HC on Progenitor Growth

PHA-M (Sigma) was diluted with supplemented DMEM. HC sodium succinate was prepared according to the manufacturer’s instructions and then diluted with supplemented DMEM. The LDA was performed as earlier by using cells from elutriation and Percoll gradient centrifugation (Fr 3/40/60 cells) with the addition of PHA or HC. When testing PHA, no HC was added to the culture. Fresh PHA (0.01 to 10 μg/mL) and HC (10^{-9} to 10^{-4} mol/L) were prepared for each experiment.

RESULTS

Composition of Cells After Percoll Separation and Elutriation of BMBC

The cell recovery in Fr 40/60 from the Percoll gradient was 11.2% of the initial BMBC layer. The average cell composition in this fraction was in agreement with our previous study: 18.7% unclassified blasts plus myeloblasts, 2.4% promyelocytes, 3.7% myelocytes plus metamyelocytes, 15.4% granulocytes, 9.1% erythroblasts, 13.7% monocytes or macrophages, and 39% lymphocytes.

For testing T cell–active agents for their direct effects on progenitors, T cells were eliminated by elutriation of BMBC cells before the Percoll gradient. After elutriation, the highest cell count was observed in the large cell fraction (Fr3), which contained about 60% of the input cells, most of them having the morphology of myelomonocytic cells. When this fraction was further separated by discontinuous Percoll gradient centrifugation, the 40%/60% layer (Fr: 3.40/60) contained 50% to 80% blasts and promyelocytes and was five- to ninefold enriched for progenitor activities over the BMBC layer. By limiting-dilution analysis, we have shown...
determined as described in the test for the wells from wells by filling the plates (ie, the cells did not sediment into suspension was uniform (ie, did not contain clumps that would be valid, it was necessary to demonstrate that the cell suspension3 did not contain only 1/3,000 to 1/10,000 T cells.21

**LDA of the Growth of BM Cells**

For the statistical approaches used in this analysis to be valid, it was necessary to demonstrate that the cell suspension was uniform (ie, did not contain clumps that would be scored as colonies) and could be distributed in the 10-μL wells by filling the plates (ie, the cells did not sediment into the wells from the additional volume above the wells during the plating). We verified the actual number of cells remaining in each well after distributing a known cell concentration into the microwell plates (Fig 1). In this experiment, the cells were plated at 1,000 cells/mL, and a typical Poisson distribution was confirmed with a peak at ten cells per well. Cells were also plated at 100 cells/mL in 180 wells, and the cell distribution was examined in the same way; the percentage of wells that contained no cells was 36% (66/180). This is close to the result predicted by the zero term of the Poisson distribution (37% negative wells). Thus, the statistical vehicles used for the analyses were appropriate.

Clones of proliferating cells were distinguished from the non-dividing cells remaining in the wells by their larger size, refractile surface by phase-contrast microscopy, and homogeneous appearance (Fig 2). Nonproliferating cells and aggregates usually disappeared by the seventh day of culture. We also investigated the optimum duration of growth for the analysis. The data obtained at day 10 showed the highest progenitor frequencies with narrowest confidence intervals in the linear regression analysis; therefore, cultures were scored on day 10. By day 14, the cells had usually degenerated, and colonies could not be scored.

Before determining the degree to which various factors influence growth of progenitors in vitro, it was necessary to establish the criteria to be used in scoring a well as positive for growth. No colonies consisting of more than eight cells were formed in wells with Fr 40/60 cells cultured without CSF or in wells with 500 purified T lymphocytes. Initially, assays were scored using multiple criteria (≥8, ≥10, ≥20, ≥30, and ≥40 cells/colony). When more than one colony was present, only the largest colony in each well was scored. When the confidence intervals were compared for each criterion, the criterion ≥10 cells was determined to be the most reliable. Calculated frequencies of progenitors cultured with 10% GCT and scored by the different criteria from eight different donors are shown in Table 1.

The estimated frequency of progenitor cells was obtained by analyzing the relationship between the number of cells plated per well and the percentage of wells found to be negative for cell growth. According to the zero term of the Poisson distribution, each well may be considered to contain an average of one precursor when 37% of a series of replicate wells are negative. In previous experiments using 10% GCT, it was shown that each criterion including ≥8, ≥10, ≥20, ≥30, and ≥40 cells/colony demonstrated single-hit kinetics,

![Image](https://www.bloodjournal.org/content/71/2/1613/F2)

**Fig 2. Examples of colonies in microwells examined by phase-contrast microscopy at day 10 of culture:** (A) ≥10 cells, and (B) ≥40 cells colonies.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of Cells/Colony*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥8</td>
</tr>
<tr>
<td>1</td>
<td>1/58 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>1/56 ± 5</td>
</tr>
<tr>
<td>3</td>
<td>1/84 ± 5</td>
</tr>
<tr>
<td>4</td>
<td>1/115 ± 14</td>
</tr>
<tr>
<td>5</td>
<td>1/32 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>1/74 ± 5</td>
</tr>
<tr>
<td>7</td>
<td>1/52 ± 7</td>
</tr>
<tr>
<td>8</td>
<td>1/40 ± 4</td>
</tr>
<tr>
<td>Mean</td>
<td>1/64</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*In each experiment, the same plates were scored five times by using criteria from ≥8 to ≥40 cells to define a colony. Precursor frequencies were determined as described in the test for each criterion. Data are given as the mean precursor frequencies for each criterion ± SD.
Fig 3. Representative results obtained from IDA of progenitors with CSFs. Each CSF was tested with three to four different donors. (A) GCT. A nonlinear response was observed in one of four donors at 1%. (B) HPCM. A nonlinear response was observed in one of four patients at 1%. (C) BCM. Nonlinear responses were observed in one of three donors at 0.5% and one of three patients at 10%. (D) Recombinant CSF: Approximately the same progenitor frequency was obtained with either 250 or 500 U/mL. Both of these results were within the confidence intervals for a parallel assay with 10% GCT. For clarity, only one line is shown. The progenitor frequency was slightly decreased with 50 and 100 U/mL, and single-hit kinetics were not observed with 10 U/mL in three donors.

did thereby indicating that only one cell type was limiting in the culture system. Thus, no helper or suppressor accessory cell function affecting progenitor growth was observed under these conditions.

Screening of CSFs on Progenitor Growth

GCT. In the absence of a source of CSF, no colonies were observed. In most experiments, GCT supported progenitor cell growth with single-hit kinetics at various concentrations (1% to 20%); however, the highest frequency was observed with a concentration of 10%. The dose response of progenitor growth demonstrated normal biologic variation from donor to donor, but a representative experiment is demonstrated in Fig 3A. When progenitor frequency was compared with that of other CSFs, GCT usually yielded the highest frequency (Table 2). The colony-forming cells harvested from cultures containing GCT showed both myeloid (86%) and macrophage-monocyte (14%) differentiation (Table 3).

HPCM. A similar trend was observed in cultures containing HPCM. Progenitor growth with single-hit kinetics was supported by HPCM, and maximum progenitor yields were observed at 5% or 10% concentration (Fig 3B). Lower

Table 2. Progenitor Frequencies in LDA Cultured With Different CSFs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>10% GCT</th>
<th>10% HPCM</th>
<th>10% PHA-LCM</th>
<th>10% BCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/99</td>
<td>1/124</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>1/143</td>
<td>1/176</td>
<td>1/225</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>1/39</td>
<td>1/167</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>1/59</td>
<td>1/57</td>
<td>1/118</td>
<td>1/99</td>
</tr>
<tr>
<td>5</td>
<td>1/44</td>
<td>1/28</td>
<td>1/71</td>
<td>1/57</td>
</tr>
<tr>
<td>6</td>
<td>1/132</td>
<td>ND</td>
<td>ND</td>
<td>1/171</td>
</tr>
</tbody>
</table>

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<td>1/39</td>
<td>1/167</td>
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<td>1/132</td>
<td>ND</td>
<td>ND</td>
<td>1/171</td>
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In each experiment, cells from a single marrow sample were stimulated with the indicated sources of CSFs, and the progenitor frequencies were determined as described in the text by using the criterion ≥10 cells/ colony.

Table 3. Effect of Different CSFs on Progenitor Maturation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Promye/Blast*</th>
<th>Juvenile</th>
<th>Polys</th>
<th>Macrophage/Monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCT</td>
<td>50</td>
<td>14.5</td>
<td>21.3</td>
<td>14.3</td>
</tr>
<tr>
<td>HPCM</td>
<td>75</td>
<td>9.1</td>
<td>11.2</td>
<td>4.4</td>
</tr>
<tr>
<td>PHA-LCM</td>
<td>53</td>
<td>11</td>
<td>8.3</td>
<td>28</td>
</tr>
<tr>
<td>BCM</td>
<td>55</td>
<td>8.2</td>
<td>20.3</td>
<td>16.3</td>
</tr>
</tbody>
</table>

BM mononuclear cells were stimulated with various sources of CSF as described in the footnote to Table 2, representative wells were harvested at day 10 and pooled, and differential morphological examinations were performed. The average results of three to four experiments are shown.

*Promye/Blasts include immature blasts, myeloblasts and promyelocytes. Juvenile myeloid cells include myelocytes and metamyelocytes. Polys are polymorphonuclear leukocytes.
levels appeared to be limiting, and in one donor, the response to 1% HPCM deviated from linearity.

**BCM.** Cell growth with single-hit kinetics was also usually observed with BCM (Fig 3C). The maximum progenitor yield was always obtained with 1% BCM. A nonlinear response was seen in one donor at the lowest concentration (0.5%) and in one donor at the highest concentration (10%). Because of the ability of a 1% concentration of BCM to yield about the same progenitor frequency obtained with other CSFs, the synergistic effect with GCT was evaluated by adding 1% BCM to 10% GCT in the culture. The progenitor yield remained almost the same as with GCT alone (data not shown).

**Recombinant CSF.** In three experiments, cultures containing 250 or 500 U/mL recombinant CSF yielded a linear response with approximately the same frequency of progenitors as 10% GCT. A slightly decreased frequency was obtained at 50 and 100 U/mL, and 10 U/mL failed to yield a linear response. Thus, as in experiments with GCT, HPCM, and BCM, the CSFs directly stimulated progenitor growth independent of accessory cell populations.

**PHA-LCM.** In some cases, PHA-LCM supported cell growth with single-hit kinetics, although the progenitor frequency was usually low compared with the frequency obtained by 10% GCT (Fig 4). No significant change in the progenitor frequency was observed after the addition of erythropoietin, nor was there any development of erythroid cultures. In some cases, however, the presence of suppressor or helper effects was evident as deviations from linearity. Thus, the results of progenitor growth with PHA-LCM were unpredictable. In wells containing PHA-LCM, aggregates of cells were often observed, which suggested the presence of residual lectin. Consequently, the effect of PHA alone was investigated.

**Effect of PHA and HC on T Cell–Depleted BM Cells**

**PHA.** As shown in Fig 5, PHA-containing medium showed suppressive action on progenitor growth at levels as low as 0.1 µg/mL, whereas at least 5 µg/mL of PHA was required to stimulate ³H-thymidine uptake with lymphocytes (data not shown). In cultures containing higher concentrations of PHA, cells tended to form aggregates. These were distinguishable from myelomonocytic cells colonies by morphology.

**HC.** HC was found to have an enhancing effect on progenitor growth at concentrations from $10^{-7}$ to $10^{-4}$ mol/L (Fig 6). The addition of HC at this concentration increased the progenitor frequency with single-hit kinetics. A concentration of $10^{-4}$ mol/L always resulted in suppression, and the effect of a $10^{-5}$ mol/L concentration was subject to variable responses with different donors. Colonies from cultures without HC tended to be smaller and showed signs of early degeneration.

**Morphological Evaluation**

Evaluation of cells from wells containing colonies by cytochemistry with myeloperoxidase, AS-D-chloroacetate esterase, and nonspecific esterase staining demonstrated a myelomonocytic lineage (data not shown). Colonies from cells cultured with different CSFs were also evaluated morphologically. No significant difference of CSFs on progenitor
can be accomplished because LDA can identify helper or colony assays (eg, 8 cells or 40 cells) can also be used in of progenitors. Most of the criteria used in methylcellulose of cells per well results in an apparent decreasing frequency of progenitor cells responsive to stimulatory factors in a given sample that contains mixed cell populations, it is important to exclude the possible effects of other cell types such as monocytes and T lymphocytes on progenitor growth. This can be accomplished because LDA can identify helper or suppressive effects. When the number of cells plated per well is plotted v the log of the percent nonresponding wells, linearity indicates that clonal growth of progenitors is unaffected by the cells present and only dependent on the presence or absence of a progenitor. Deviation from linearity indicates the presence of accessory cell populations that have helping or suppressing influences on progenitor growth. In the presence of suppressor cells, plating increasing numbers of cells per well results in an apparent decreasing frequency of progenitors. Hence, we adapted LDA to liquid suspension of colonies (CFU-GM plus BFU-E plus CFU-GEMM) compared with PHA-LCM (data not shown). For clonal culture of primitive hematopoietic precursors, PHA-LCM was considered to be essential. However, this does not seem to be true in Dexter-type long-term BM culture in which primitive progenitors can be enumerated. Fewer mixed colonies were obtained with 10% PHA-LCM than with 7.5% in methylcellulose culture.6 Thus, our results indicate that the crude preparation of PHA-LCM cannot be used reproducibly in LDA.

In previous reports, PHA was added to agar or methylcellulose cultures to study the effect of activated T cells on progenitor growth. The addition of PHA was thought to promote burst colony formation. In agar culture systems, the addition of PHA alone to T cell–depleted marrow cells was reported to have had no effect on GM progenitor growth at a concentration of 20 μg/mL.6 Additionally, PHA-stimulated conditioned medium has been used to stimulate the growth of progenitor cells in semisolid and liquid suspension cultures.6 In LDA, however, a suppressive effect of PHA on progenitor growth was observed at levels as low as 0.1 μg/mL.

HC prevents the growth and function of T cells44 and increases marrow granulocyte production in long-term BM culture.44 However, reports on the effect of glucocorticosteroids on granulopoiesis in short-term culture systems have been contradictory. Suda et al45 reported that the addition of HC (10^-6 mol/L) to T cell– and phagocyte-depleted BM cells increased the number of granulocyte colonies but decreased macrophage colonies in agar culture. Enhanced formation of granulocyte colonies was also observed after the addition of HC in agar45 and in a diffusion chamber culture system.46 Slovick et al,46 however, reported that the addition of 10^-6 mol/L HC did not significantly change the total maturation was observed, as shown in Table 3. Erythroid maturation was not observed, even in the presence of erythropoietin.

**DISCUSSION**

LDA is a quantitative method to determine the frequency of progenitor cells responsive to stimulatory factors in a given population of cells. LDA has proved to be a useful measure for evaluating the factors regulating growth of immunocompetent cells.36 To analyze responding GM precursors in a sample that contains mixed cell populations, it is important to exclude the possible effects of other cell types such as monocytes and T lymphocytes on progenitor growth. This can be accomplished because LDA can identify helper or suppressive effects. When the number of cells plated per well is plotted v the log of the percent nonresponding wells, linearity indicates that clonal growth of progenitors is unaffected by the cells present and only dependent on the presence or absence of a progenitor. Deviation from linearity indicates the presence of accessory cell populations that have helping or suppressing influences on progenitor growth. In the presence of suppressor cells, plating increasing numbers of cells per well results in an apparent decreasing frequency of progenitors. Hence, we adapted LDA to liquid suspension of GM progenitor cultures to establish an assay for the evaluation of factors regulating hematopoiesis. Murine hematopoietic progenitors have been assayed in microwell cultures after isolation by fluorescence-activated cell sorting.35,36 When using an increase in cell number as a response to CSF, accessory cell effects could not be ruled out.36 LDA of murine BM and spleen GM progenitors without definition of the criteria for clonal growth was hampered by highly variable confidence intervals.36

In semisolid culture systems, various criteria have been used to define clonal growth, and the biologic implications of these criteria have not been established. In this study, we have evaluated different criteria for defining clonal growth of progenitors. Most of the criteria used in methylcellulose colony assays (eg, ≥8 cells or ≥40 cells) can also be used in our system. Among them, the criterion ≥10 cells appeared to provide the most consistent results and was used to compare the effect of factors on progenitor growth. We speculate that the larger colonies may represent more primitive progenitors with a greater proliferation potential, whereas the smaller colonies may represent more differentiated cells.

The different responses to CSFs observed in this study suggest that the LDA might measure a different set of progenitors than the semisolid colony assays. In LDA, the best progenitor yield was almost always obtained from cultures containing 10% GCT. BCM and recombinant CSF produced almost the same number of progenitors, but in some instances, PHA-LCM did not. On the contrary, the use of GCT in methylcellulose usually yielded a smaller number of colonies (CFU-GM plus BFU-E plus CFU-GEMM) compared with PHA-LCM (data not shown). For clonal culture of primitive hematopoietic precursors, PHA-LCM was considered to be essential. However, this does not seem to be true in Dexter-type long-term BM culture in which primitive progenitors can be enumerated. Fewer mixed colonies were obtained with 10% PHA-LCM than with 7.5% in methylcellulose culture.6 Thus, our results indicate that the crude preparation of PHA-LCM cannot be used reproducibly in LDA.

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LIMITING-DILUTION ANALYSIS OF MARROW PROGENITORS

number of GM-CFC in agar culture. Cheng et al. also noted that the growth of granulocytic colonies in agar was not affected by the presence of up to $10^{-4}$ mol/L HC. In this study, HC was shown for the first time to have a direct enhancing function on progenitor cell growth in short-term suspension cultures at concentrations of $10^{-7}$ to $10^{-6}$ mol/L. Cells cultured with HC also formed larger colonies and tended to be more homogeneous and round, similar to findings reported by Barr et al. We found the use of HC to prevent T cell growth in our assay system to be critical because the possible concomitant growth of T cells may be mistaken for or interfere with GM progenitor growth. The precise action of HC on progenitor cell growth is still unclear; however, LDA provides an approach for further investigation.

Optimum concentrations of CSFs for progenitor growth have been previously reported that suggest that promotion of clonal growth by conditioned media is regulated by a balance between stimulators and inhibitors. Thus, the decrease in progenitor frequencies in wells cultured with 1%, 5%, and 20% GCT may be due to a deficiency of CSF at lower concentrations and to the presence of growth-inhibitory substances at higher concentrations. In this respect, LDA has proved to be a reasonable approach for investigation of the mechanism of action of these factors.

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Limiting-dilution analysis of the effects of colony-stimulating factors, phytohemagglutinin, and hydrocortisone on hematopoietic progenitor cell growth

Y Takaue, CL Reading, AJ Roome, KA Dicke, S Tindle, M Chandran and B Devaraj