Induction of Proliferation of Purified Human Myeloid Progenitor Cells: A Rapid Assay for Granulocyte Colony-Stimulating Factors

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The proliferation and differentiation of granulocyte and monocyte progenitor cells (CFU-C) in vitro is dependent on the presence of a group of closely related glycoproteins termed colony-stimulating factors (CSF). In order to investigate the interaction of these factors with CFU-C, we purified CFU-C from the peripheral blood of chronic myeloid leukemia patients with an immune rosette technique using specific monoclonal antibodies (mean 74-fold enrichment, 45% cloning efficiency). Colony formation by purified CFU-C demonstrated an absolute dependence on an exogenous source of CSF. Liquid culture of small aliquots of enriched CFU-C with CSF-containing medium resulted in a rapid, time- and concentration-dependent induction of DNA synthesis as measured by $^3$H-thymidine incorporation. This specific CSF induction of DNA synthesis by enriched CFU-C was used to develop a microassay system for CSF activity. CSF activity could be reproducibly quantitated in 24–48 hr. The proliferating cells in this assay system were shown to be myeloid progenitor cells by examining the morphology of their progeny and by determining the surface antigen phenotypes of the responding cells (la+, T3, B1, Mo1). This microassay provides a quantitative assessment of CSF activity that may be useful in the purification of human CSF and in the generation of monoclonal antibodies to CFU-C surface structures.

GRANULOCYTES AND MONOCYTES in the peripheral blood are derived from bone marrow precursor cells committed to myeloid differentiation. These committed progenitor cells can be cultured in vitro in semisolid medium (colony-forming unit, CFU-C) to produce colonies of mature cells when exposed to appropriate growth factors (colony-stimulating factors, CSF). CSF appear to be critical for viability, proliferation, and differentiation of CFU-C in vitro and are produced by activated T cells, monocytes, and possibly other cells. Although small quantities of both human and murine colony-stimulating factors have been highly purified, little is known about their mechanism of action on CFU-C cells, largely because it has not been possible to prepare purified populations of CFU-C. In an effort to begin to study the interaction of CSF with CFU-C, we have recently developed a technique to isolate large numbers of myeloid progenitor cells from the peripheral blood of chronic myeloid leukemia (CML) patients. CML peripheral blood contains an increased number of CFU-C compared to normal peripheral blood, and these cells differentiate into functional granulocytes and monocytes in vivo. The CFU-C purification procedure uses specific monoclonal antibodies to deplete T lymphocytes, B lymphocytes, null lymphocytes, basophils, monocytes, and maturing myeloid cells. The purified CFU-C are therefore separated to a large degree from endogenous cells capable of producing CSF or other regulatory molecules. After purification, approximately 50% of cells will form a myeloid colony or cluster in agar, and there is an absolute requirement for CSF for colony formation. Approximately 90% of the purified cells have the morphological appearance of myeloblasts, with the remainder of cells being primarily promyelocytes.

The results presented here describe the use of these purified myeloblasts to monitor the induction of progenitor cell proliferation by CSF. Proliferation is assessed in a highly reproducible, simple microassay by measuring uptake of $^3$H-thymidine ($H$-TdR) by $10^4$–$10^5$ cells at 24–72 hr of exposure to CSF. This specific induction of myeloblast proliferation by CSF provides a rapid, highly sensitive, and readily quantitated assay for human colony-stimulating factors.

MATERIALS AND METHODS

CML Cells

Peripheral blood samples (5–10 ml) were obtained from CML patients undergoing initial diagnostic testing or therapeutic leukapheresis. All patients met standard criteria for the diagnosis of stable phase CML, and chromosome analysis in each case revealed the Ph+ chromosome. No patient was currently receiving chemotherapy. All blood and bone marrow specimens were obtained following guidelines approved by the institutional review boards of the Dana-Farber Cancer Institute and University Hospital. All patients had white cell counts >$10^5$/liter, and ≤1% blasts on a differential count. Blood was collected into sterile heparinized syringes and mononuclear cells separated by sedimentation on Ficoll-Hypaque gradients. Interface cells were washed twice in minimal essential medium (GIBCO, Grand Island, NY) containing 2-5% pooled human AB serum (MEM-AB wash). In all cases, cells were cryopreserved in vapor phase of liquid nitrogen in 10% dimethylsulfoxide and 20% heat-
inactivated fetal bovine serum (FBS) until use. These samples were thawed in the presence of deoxyribonuclease I, 100 μg/ml (Worthington Biochemicals, Freehold, NJ), to minimize cell agglutination.

**Purification of CFU-C**

CFU-C were considered to include those cells forming clusters (8-40 cells) plus those cells forming colonies (>40 cells) at day 7 in an agar assay. CFU-C were purified by a modification of a previously described technique. CML peripheral blood mononuclear cells were simultaneously depleted of T cells, B cells, null lymphocytes, basophils, monocytes, and granulocyte lineage cells (as immature as the promyelocyte) using murine monoclonal antibodies specific for each type (see below). Cells binding antibody were then removed by the formation of rosettes with sheep erythrocytes coated with affinity-purified rabbit anti-mouse immunoglobulin. Rosetted cells were separated from nonrosetted cells by a Ficoll-Hypaque gradient. The interface cells (antibody negative) contain 0.5%-1% of the starting number of cells and 75%-99% of the total number of CFU-C cells. Overall yield of CFU-C in multiple experiments ranged from 45% to 99%.

**Monoclonal Antibodies**

A panel of monoclonal antibodies was selected to specifically and simultaneously deplete non-CFU-C cells in CML PBL using the immune rosette technique. Granulocytes, monocytes, and their precursors (as early as the promyelocyte) were identified with anti-Mo1 and anti-MY8 antibodies. Anti-MY4 was also added to improve depletion of monocytes. Large granular lymphocytes and CML basophils were identified by 31C6 (T. Hencord and J. Ritz, unpublished). Anti-T11 (sheep erythrocyte receptor) and anti-B1 were used to deplete T cells and B cells, respectively. Non-T, non-B lymphocytes were identified using anti-N901 and Mo1. All antibodies were used as diluted ascites fluid.

**Immune Rosette Formation**

Rabbits were immunized with mouse Ig, and rabbit anti-mouse Ig was prepared by passing serum sequentially over a human Ig affinity column (to remove cross-reacting antibodies) and a mouse Ig affinity column. Purified rabbit anti-mouse Ig was then eluted from the second column with 1 M glycine buffer, pH 2.0, extensively dialyzed against 0.16 M sodium chloride, and stored at −20°C until use. Rabbit anti-mouse Ig was attached to erythrocytes as follows: sheep erythrocytes (Microbiological Associates, Walkersville, NC) were washed 5 times with 0.9% NaCl, and 0.5 ml packed erythrocytes were added to 0.5 ml of chlorolamidine (Fisher Scientific, Pittsburgh, PA), 1 mg/ml in 0.9% NaCl and 0.5 ml rabbit anti-mouse Ig (1 mg/ml in BBS). All solutions were passed through a 0.45-μm filter unit (Millipore Corp., Bedford, MA) prior to use. The cell suspension was gently mixed by shaking for 7 min at 23°C. The reaction was terminated by addition of 10 ml cold phosphate-buffered saline (BBS), and after centrifugation, the Ig-coated erythrocytes were washed 5 times with 0.9% NaCl. These cells are stable when stored as a 10% suspension at 4°C for up to 7 days.

Immune rosettes were formed by incubating 0.5-10 x 10⁶ CML PBL (cryopreserved) with 1 ml of minimal essential medium containing 2.5% human AB serum (MEM-AB) with 1:100 dilutions (from ascites) of anti-Mo1, MY8, MY4, B1, T11, N901, and 31C6 for 30 min at 4°C. Unbound antibody was removed by two wash steps, and 0.75 ml of a 10% Ig-coated erythrocyte suspension was added to 10⁶ monoclonal antibody-treated cells. The mixture was pelleted (300 g, 10 min) and then incubated at 4°C for 30 min. The red cell-leukocyte mixture was then vigorously suspended with a Pasteur pipette, and the frequency of rosetted leukocytes ascertained by microscopic examination. Rosetted cells were then separated from non-red cell-bearing cells by Ficoll-Hypaque density gradient sedimentation (1.077 g/ml). Interface (antibody negative) cells were collected in a sterile pipette, washed, and suspended in Iscove's modified Dulbecco's MEM, 20% FBS, for colony assay. Cells were counted in a hemacytometer and viability assessed by trypan blue exclusion. Viability was generally >98% in all experiments.

Our previous experiments utilized a second rosetting step to select La+ cells from the initial rosette-negative cells. This was necessary because of the contamination with basophils. The addition of the basophil-reactive antibody, 31C6, to the initial panel has resulted in equivalent purity of progenitor cells (>90% blasts, with cloning efficacy of approximately 50%) in a single step.

**CSF-Induced Proliferation Assay**

A quantity of 10⁵-10⁶ purified CML PBL myeloblasts were cultured in 150 μl of RPMI 1640 medium (GIBCO) containing 10% FBS and varying concentrations of CSF-containing conditioned medium for 24-96 hr in round-bottom microtiter plates (96 well, Linbro). Four hours prior to cell harvest, 50 μl of RPMI 1640 containing 1 μCi ³H-TdR (1.9 Ci/mole, Schwarz-Mann, Spring Valley, NY) was added. Cells were harvested on an automatic cell harvester (Microbiological Associates, Bethesda, MD) and ³H-TdR incorporation was measured in a Packard scintillation counter (Packard Instrument Co., Downer’s Grove, IL). Assays were run in triplicate or quadruplicate, and results are expressed as mean ± standard deviation. In some experiments, cells were washed free of unbound CSF after varying time periods by pelleting the cells in the microtiter plates (300 g, 5 min), removing the medium, two more wash steps, followed by cell suspension in medium without CSF. In some experiments, cells were exposed to phytohemagglutinin or pokeweed mitogen (Difco Laboratories, Detroit, MI).

**CSF Colony Assay**

CSF was quantified by using a colony assay that has been previously described. Aliquots (0.2 ml) of media to be tested for the presence of CSF were distributed by pipette into 35-mm polystyrene petri dishes and then resuspended in 1-ml volumes of supplemented modified Eagle's medium containing 0.5% agar. Buoyant, nonadherent human marrow cells, fractionated by Ficoll-Hypaque centrifugation and nylon fiber chromatography, were resuspended at a concentration of 1.5-3.0 x 10⁶ cells/ml in the same medium containing 0.3% agar and then added in 1-ml volumes to each culture dish onto the previously prepared underlayers. Maximal stimulation of CFU-C proliferation (or 100% CSF activity) was defined in each experiment as the number of colonies that developed in 3-6 control cultures stimulated by feeder layers containing 10⁶ peripheral blood leukocytes obtained from healthy volunteers. All cultures were incubated for 13 days. In maximally stimulated control cultures, colony growth varied from 30 to 250 colonies per 2 x 10⁶ marrow cells. In unstimulated control cultures plated in each experiment, colony growth was not observed. Colonies (>40 cells) were counted using an inverted microscope. Marrow samples were obtained from patients free of hematologic malignancies. Enriched CML CFU-C were plated at 1-2.5 x 10⁶ cells/ml for colony assay. Erythroid colonies were assayed by the method of Clark and Houseman using 1 U/ml erythropoietin (Connaught Step III, Willowdale, Ontario).

**Sources of CSF**

CSF-containing conditioned media were obtained from several sources. Most experiments were performed using GCT medium
(GIBCO) as a source of CSF. This medium stimulates granulocyte, monocyte, and eosinophil colony growth. Media conditioned by endotoxin-stimulated monocytes and lectin-stimulated lymphocytes were also used. For the latter, venous blood from healthy volunteers was collected in dilute preservative-free heparin and fractionated by Ficoll-Hypaque centrifugation followed by nylon fiber chromatography. Lymphocytes prepared in this manner were incubated at a cell concentration of 2 x 10⁵/ml in McCoy's 5A medium, containing 50 μg/ml streptomycin, 50 IU/ml penicillin G, 100 μg/ml concanavalin A (Pharmacia Chemicals, Uppsala, Sweden), and 0.5 mg/ml polyethylene glycol (P-3640, Sigma Chemical Co., St. Louis, MO) for 6 days at 37°C in a humid incubator containing 5% CO₂. Approximately 500 ml of the conditioned medium was then concentrated 50-fold using PM-10 and B-15 Amicon filters in sequence. The concentrated medium was stored at −80°C.

**Partial Purification of Myeloid Progenitor Cells**

Three milliliters of concentrated lymphocyte conditioned medium was applied to an 80 x 1.75 cm column of Sephadex S-200 (Pharmacia Chemicals) and 60 5-ml fractions were collected at 4°C using 0.02 M phosphate-buffered 0.15 M saline, pH 7.4 (PBS), as the running buffer. The column was calibrated using dextran blue, ferritin, catalase, aldolase, bovine serum albumin, ovalbumin, and phenol red as molecular weight markers. Each fraction was sterilized using a 0.22-μm Millex-GV filter (Millipore) to minimize protein loss and was stored at −80°C pending CSF assay.

**Complement Lysis of Purified Progenitor Cells**

Purified CML PBL CFU-C (10⁵) were incubated with 100 μl of individual lytic antibodies (anti-Ia, Mol, B1, T3, β₂, microglobulin, or control antibody diluted 1:100 in RPMI 1640) for 30 min at 4°C. Fifty microliters of prescreened rabbit serum was then added as a source of complement, followed by an additional incubation at 37°C for 1 hr. The cells were then washed twice as described above and were suspended in 150 μl of RPMI 1640 containing sufficient GCT medium to provide maximum stimulation for a 48-hr assay (5% v/v). At 44 hr of incubation, the cultures (in quadruplicate) were pulsed with ³H-TdR, and the cells were harvested 4 hr later. Percent inhibition of proliferation was calculated as:

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1 \rightarrow \frac{\text{cpm (antibody + complement)}}{\text{cpm (complement alone)}} \times 100.
\]

**RESULTS**

**Partial Purification of Myeloid Progenitor Cells From CML Peripheral Blood**

Simultaneous depletion of granulocytes, monocytes, basophils, T cells, B cells, "null" cells, and immature myeloid cells using a panel of monoclonal antibodies specific for each cell type and immune rosette formation resulted in a 49–110-fold purification of myeloid colony- and cluster-forming cells in a single step (mean 74-fold in 6 experiments). In these experiments, 30%–62% of the enriched cell population could proliferate in agar to form either a myeloid colony or cluster at day 7 (mean 45% ± 11%, 6 experiments). Confirmation of the myeloid nature of the colonies was obtained in each case by in situ staining of whole CFU-C agar cultures for chloroacetate esterase activity, as previously described. The majority of cells proliferating in agar formed clusters of 8–40 cells at day 7 (mean 33% ± 13% of total cells), whereas 12% ± 6% of total cells formed a colony (>40 cells). A proportion of the remaining cells formed smaller clusters of myeloid cells (2–7 cells). In the absence of CSF, no colonies or clusters were formed. In most experiments, day 13 CFU-C were also assayed and were similarly enriched. Erythroid colonies were formed by 3%–8% of cells (mean 5.6%, 3 experiments) when assayed separately in erythropoietin-containing medium.

**Proliferation of Purified CFU-C Preparations in Liquid Culture**

In order to examine the effects of CSF-containing conditioned medium in liquid suspension culture, aliquots of purified CFU-C were cultured at 5 x 10⁵ cells/ml in the presence or absence of CSF-containing medium (GCT). Cell number and the percent of differentiated cells were then sequentially determined. As shown in Table 1, there was an eightfold increase in cell number in cultures containing CSF after 7 days of culture. This increase in cell number was due to the
generation of morphologically recognizable mature granulocyte lineage cells. In contrast, there was no increase in either total cell number or the number of differentiated cells in the absence of CSF. Lymphocytes and erythroid cells were not observed. These results demonstrate that, under the conditions of this assay, CSF-containing conditioned medium induces myeloid cell proliferation and that, by day 7, the majority of cells are differentiated granulocyte lineage cells.

CSF-Induced DNA Synthesis in Purified Myeloid Progenitor Cells

Aliquots of $10^4$–$10^5$ purified CML myeloblasts were cultured in triplicate in microtiter plates with varying concentrations of GCT conditioned medium as a source of CSF for 48 hr (Fig. 1). Four hours prior to cell harvest, 1 μCi of $^3$H-TdR was added to each culture. Incorporation of $^3$H-TdR during this 4-hr pulse was used as a measurement of DNA synthesis. In a representative experiment shown in Fig. 1, in the absence of added CSF, "background" $^3$H-TdR incorporation ranged from 200 cpm ($10^3$ cells/well) to 1,250 cpm ($10^4$ cells/well). The addition of CSF resulted in a dose-dependent increase in $^3$H-TdR incorporation at all cell concentrations, with as little as 1.3% GCT medium (1 μl/150 μl culture). After 48 hr of culture, maximum stimulation occurred, with 6%–8% GCT medium. The lack of DNA synthesis in cultures without added CSF is consistent with our previous observation that colony and cluster formation (dependent on cell proliferation) of these purified CFU-C required added CSF.  

The results shown in Fig. 1 indicate that $^3$H-TdR incorporation is directly related to CSF concentration at 48 hr of culture. Figure 2 shows the results of culture of $10^4$ cells for 24, 48, or 72 hr, with varying concentrations of CSF (GCT conditioned medium). As shown, at concentrations of CSF greater than 0.3%, there was a time-dependent increase in $^3$H-TdR incorporation. Similar studies have been performed using other sources of CSF, including medium conditioned by lymphocytes stimulated by concanavalin A and by endotoxin-stimulated monocytes. In each case, there was a dose- and time-dependent stimulation of $^3$H-TdR incorporation very similar to the curves shown in Fig. 2 for GCT medium. Compared to 1% GCT medium, 1% concentrations of concanavalin A or endotoxin-stimulated media resulted in 92% and 65%, respectively, of the $^3$H-TdR incorporation induced by GCT at 48 hr. Exposure of the cells to phytohemagglutinin (4 μg/ml) or pokeweed mitogen (50 μg/ml) did not induce incorporation of $^3$H-TdR at 48 hr (data not shown).

Our previous studies have demonstrated that very few colony- or cluster-forming cells are found in the rosette-positive cell fraction. It would be anticipated, therefore, that exposure of these cells (primarily immature granulocytes with smaller numbers of lymphocytes and monocytes) to CSF would not result in an increase in $^3$H-TdR incorporation. To test this, $10^4$ rosette-positive cells were cultured with varying concentrations of GCT medium (0%–7%) for 48 hr. Background incorporation (0% GCT medium) was approximately 100 cpm. Addition of CSF did not result in any significant increase in $^3$H-TdR incorporation (145 ± 61 cpm) with any concentration of GCT medium tested (0%–7% at 48 hr). These results confirm our observation that most myeloid cells capable of proliferation lack reactivity with any of the antibodies used in purification, and therefore remain in the rosette-negative fraction.

Characterization of the Proliferating Cells in Purified CFU-C Populations

In order to further characterize the proliferating cells, antibody lysis experiments were done to determine...
the surface antigen phenotype of the cells responding to CSF-containing conditioned medium. Aliquots of purified CFU-C (5 x 10^4 cells in quadruplicate) were treated with antibody and complement in microtiter wells, as described in Materials and Methods, prior to culture in GCT-containing medium for 48 hr. ^3H-TdR incorporation during a 4-hr pulse was then determined (Table 2). As shown in Table 2, lysis of Ia^- cells completely abrogated subsequent CSF-induced proliferation, whereas removal of T cells (anti-T3), B cells (anti-B1), or monocytes, large granular lymphocytes, and maturing granulocyte lineage cells (anti-Mo1) had no effect. Anti-beta2 microglobulin was used as a positive control. Thus, the cells induced to proliferate in this assay system have the phenotype of myeloid progenitor cells (Ia', T3', B1', Mo1'). Further, these results demonstrate that proliferation of T cells, B cells, monocytes, or myeloid cells more mature than the promyelocyte (Ia antigen is lost in myeloid differentiation at the level of the promyelocyte) does not contribute to the ^3H-TdR incorporation measured in the assay. Approximately 90% of the cells in multiple preparations were lysed by treatment with anti-Ia and complement as estimated by trypan blue exclusion.

Comparison of CSF Microassay With Colony Bioassay

In order to investigate the utility of the microassay in the purification of human CSF, conditioned medium from concanavalin A-stimulated peripheral blood leukocytes was chromatographed on a Sephadex S-200 column equilibrated in PBS. Aliquots of column fractions were tested for CSF activity in a 13-day colony assay (200 pl) and in a 48-hr microassay (10 pl). Single, overlapping peaks of CSF activity were detected by each assay (Fig. 3).

| Table 2. Surface Antigen Phenotype of Proliferating Cells in Partially Purified CFU-C Preparations |
|-----------------|-----------------|-----------------|
| **CSF** | **Antibody** | **% Inhibition** |
| | | **^3H-Thymidine Incorporation (cpm)** |
| - | - | 352 ± 51 |
| + | - | 6,129 ± 349 |
| + | + | 5,325 ± 691 |
| + | Control | 5,567 ± 418 |
| + | Ia | 523 ± 213 |
| + | T3 | 5,590 ± 971 |
| + | B1 | 5,984 ± 867 |
| + | Mo1 | 5,214 ± 650 |
| + | Beta2 microglobulin | 219 ± 59 |

Aliquots (10^4) of purified CML CFU-C were treated with antibody and complement (C) as described in Materials and Methods, followed by incubation with CSF-containing medium (5% GCT) for 48 hr. ^3H-TdR incorporation was determined after a 4-hr pulse.

Continuous Exposure to CSF Is Required for Maximum Proliferation

Aliquots of purified CFU-C were exposed to CSF-containing conditioned medium for 0 min, 5 min, 4 hr, or 24 hr, after which unbound CSF was removed by washing the cells repeatedly. ^3H-TdR incorporation during a 4-hr pulse was then determined at 24, 48, 72, and 96 hr (Fig. 4 shows a representative experiment). Exposure of purified CFU-C to CSF for as little as 5 min led to a transient, reproducible burst of proliferation (at 120 hr, incorporation was equal to background). Exposure to CSF for 4 hr resulted in slightly
higher $^{3}$H-TdR incorporation at 24 hr, but similar values thereafter. In contrast, continuous exposure to CSF resulted in a steady increase in the rate of DNA synthesis, as expected.

**DISCUSSION**

The development of an in vitro colony assay for myeloid progenitor cells has led to several lines of evidence that suggest that the interaction of CSF with CFU-C is a critical event in the regulation of myeloid differentiation. The experiments of Pluznik and Sachs and Bradley and Metcalf established that murine CFU-C would proliferate and differentiate in semi-solid medium when exposed to colony-stimulated factors. Although a number of factors have since been identified that can modify the size or number of colonies that are produced, CFU-C do not proliferate in the absence of CSF.

However, despite the important role played by CSF in hematopoiesis, very little is known about the biochemical events associated with CFU-C proliferation. This is due to the difficulty in purifying these factors, particularly human CSF, and the lack of purified populations of progenitor cells. We have previously described an immunologic technique to rapidly purify large numbers of CFU-C from the peripheral blood of CML patients. The results presented here show that these purified CFU-C are induced to proliferate in liquid culture in response to CSF and that this proliferation can be assayed by measuring $^{3}$H-TdR incorporation into DNA, thus providing a rapid, quantitative, and specific microassay system for human CSF that provides results in as little as 24 hr. The sensitivity of this assay appears to be high, as the CSF in less than 1 $\mu$l of several commonly used conditioned media can be readily detected. CML peripheral blood cells can be cryopreserved until needed, and the purification procedure is rapid. Sufficient purified CFU-C can be prepared in about 2 hr for 1,000–5,000 CSF assays, using 1,000 cells per assay. This microassay system can therefore be used to supplement standard colony assays when attempting to purify human CSF, having the advantages of speed, sensitivity, and ease of performing large numbers of assays at one time.

There have been a number of previous attempts to develop rapid CSF assays, including efforts to measure CSF-induced $^{3}$H-TdR incorporation in unseparated bone marrow cells. The use of whole bone marrow preparations, however, has been associated with a number of problems, including high background DNA synthetic activity, lack of evidence that CFU-C were the major cells incorporating thymidine, requirement for large numbers of cells, long processing of samples, and the concern that accessory cells capable of producing CSF or inhibitory factors were present in significant numbers. For example, Horak and colleagues showed that mouse lung conditioned medium stimulated mouse bone marrow cells to incorporate $^{3}$H-TdR in a dose-dependent fashion. However, the assay requires 5 days of CSF exposure, and thus offers little advantage over a 7-day colony assay. Lusis and Koeffler described the use of the AML cell line, KG-1, to assay CSF. In the presence of CSF-containing conditioned medium, KG-1 cells incorporated about 2.5-fold more $^{3}$H-TdR than background, and a rapid quantitative assay was developed. Although the use of cultured cells as targets is a considerable advantage, KG-1 cells do not require exogenous CSF for growth, and therefore, background $^{3}$H-TdR incorporation tends to be high.

The use of purified CFU-C solves many of these problems. Burgess has reported a rapid murine CSF assay system that utilized CFU-C highly purified from fetal liver by a negative selection procedure using fluorescence-activated cell sorting. Like the CFU-C used in the assay described here, endogenous CSF-secreting cells have been depleted, and the fraction of cells that will proliferate to form myeloid colonies or clusters is high. Small numbers of cells (50–200) were suspended in CSF-containing medium, and the number of cells present in each well was counted manually using an inverted microscope at 0 and 48 hr of culture. The increase in cell number was shown to provide a sensitive dose-dependent assay for murine CSF. The assay described here is potentially more rapid, as DNA synthesis would necessarily occur prior to cell division, and also has the advantages of being highly reproducible and quantitatable. It does, however, require more cells per assay. Also, factors that might affect the transport or metabolism of $^{3}$H-TdR will interfere with this assay. This would include unlabeled thymidine present in the hypoxanthine-aminopterin-thymidine medium used to select for monoclonal antibody-producing hybridoma clones. In addition, it has not been established that CML cells will respond to all types of GM-CSF, and until the different types of human CSF have been highly purified and tested, it is possible that this assay detects only some CSF species and not others. Also, although there are no published reports that CML CFU-C, like normal CFU-C, can be induced to proliferate by any substance other than CSF, further work is necessary to confirm this.

So far, it has not been possible to purify human CFU-C from normal bone marrow or fetal liver to a sufficient degree as to result in adequate specificity in an assay such as is described here. CML peripheral
blood is particularly advantageous because the numbers of myeloid progenitor cells far outweigh the numbers of other types of progenitor cells. For example, the ratio of CFU-C (clusters and colonies) to erythroid burst-forming units (BFU-E) in these preparations is generally greater than 10:1. This is important, because many sources of CSF also contain burst-promoting activity. The morphological data in Table 1 suggest, however, that non-CFU-C progenitor cells are not contributing significantly to the proliferation observed in this assay. It may soon be possible to use CFU-C from normal human bone marrow or fetal liver as a purification procedure improve. It may also be possible to bias the assay in favor of one type of CSF by purifying subsets of CFU-C. For example, the use of purified eosinophil CFU-C may bias the assay to detect eosinophil-CSF. The majority of CML CFU-C form neutrophil colonies, with smaller numbers of monocyte and eosinophil colonies, so the assay described here primarily detects types of CSF capable of stimulating neutrophil colony growth.

The results in Fig. 4 confirm earlier observations with colony formation that CSF is required continuously for maximum proliferation, as incorporation of 3H-TdR falls off rapidly once CSF is removed. It is possible that previous studies of binding of purified CSF to whole bone marrow cells reflect binding to cells that are predominantly not progenitor cells, but rather are mature myeloid cells that still bind CSF. The highly purified progenitor cells described here can be used to directly investigate the mechanism of action of CSF, and the rapid assay will also make possible, for the first time, direct use of human CFU-C for the immunization and screening of monoclonal antibodies, specifically those potentially blocking binding of CSF to cell surface receptors.

Finally, this assay will allow investigation of factors and “accessory” cells that might modify CSF-induced proliferation of these cells. Numerous such regulatory factors and cells have been proposed, but direct interaction with CFU-C has been difficult to demonstrate. For example, it has been proposed that 13-0-tetradecanoyl phorbol acetate (TPA) could act on CFU-C to stimulate proliferation in the absence of CSF. However, our studies with purified human CFU-C suggest that any colony-promoting effects of TPA are due to indirect effects, such as the stimulation of CSF secretion by macrophages. Thus, the assay described here should provide a useful method to investigate both the mechanism of CSF-induced proliferation and the role of other factors and cells capable of modifying CFU-C proliferation.

ACKNOWLEDGMENT

The authors would like to thank Dr. Robert Todd for the use of anti-Mol monoclonal antibody, Dr. Ellis Reinherz for the use of anti-T3, Dr. Lee Nadler for anti-B1, and Drs. Thierry Hercend and Jerome Ritz for 31C6 antibody.

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