Combinations of Recombinant Colony-Stimulating Factors Are Required for Optimal Hematopoietic Differentiation in Serum-deprived Culture

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During the past few years, the genes for five human hematopoietic growth factors (HGFs) or colony-stimulating factors (CSFs) have been cloned and recombinant highly purified HGFs produced. Investigations of their biologic activities using enriched progenitor cells in methylcellulose cultures that contained fetal calf serum or human plasma or serum revealed that interleukin 3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are multipotent. Alone, they induced the development of granulocyte-, macrophage-, and granulocyte-macrophage colony-forming units (CFU-G, CFU-M, CFU-GM), eosinophil CFU (CFU-Eo), and megakaryocyte CFU (CFU-Meg); in the presence of erythropoietin (ep), erythroid burst-forming units (BFU-E), and granulocyte/erythroid/macrophage/megakaryocyte colonies (CFU-GEMM) were obtained. The other three CSFs showed lineage restriction; granulocyte CSF (G-CSF), macrophage CSF (M-CSF), and ep induced the formation of granulocyte, macrophage, and a subset of erythroid colonies, respectively.

Despite the use of highly purified recombinant CSFs and enriched progenitor cells, a major obstacle to the interpretation of these results was the inclusion of fetal calf serum in the cultures, which itself might contain CSFs or other cofactors. We therefore established serum-deprived conditions for progenitor cultures in order to re-examine the actions of the CSFs in serum-deprived conditions. Results show that both the multipotentials are inadequate stimuli of colony formation. At maximal concentrations IL-3 alone induces only 25% of the granulocyte and macrophage colony-forming units (CFU-G and CFU-M) produced by a T-cell conditioned medium that contains a mixture of CSFs. When IL-3 was added at the initiation of the cultures and erythropoietin (ep), G-CSF, or M-CSF added on day 3, almost full recovery of erythroid, granulocytic, and monocytic colonies, respectively, was obtained. Similar results were obtained with GM-CSF except that fewer erythroid colonies were recovered at high concentrations, and almost maximal CFU-M proliferation could be induced. These results show that in serum-deprived conditions, the multipotentials must be combined with lineage specific CSFs for full progenitor expression.

MATERIALS AND METHODS

Growth Factors

Human IL-3. The human IL-3 was purified to homogeneity from E. coli engineered to express high levels of this factor. The purified IL-3, which had a specific activity of $2 \times 10^2$ U/mg in the chronic myelogenous leukemia (CML) blast proliferation assay, was kindly provided by J. Seehra (Genetics Institute).

Human GM-CSF. Human GM-CSF, isolated from medium conditioned by Chinese hamster ovary (CHO) cells engineered to express high levels of the factor, was kindly provided by the Genetics Institute Pilot Development Laboratory. The homogeneous GM-CSF had a specific activity of $1-4 \times 10^4$ U/mg in the CML blast proliferation assay.

Human G-CSF. As a source of recombinant human G-CSF, we used the conditioned medium from a CHO cell line engineered to express high levels of the factor (generously provided by G. Wong and R. Kaufman, Genetics Institute). This conditioned medium was found to support half-maximal human granulocytic colony formation with normal bone marrow at a final dilution of 1:30,000.

Human M-CSF. Human M-CSF was partially purified from medium conditioned by a CHO cell line engineered to produce the human factor (kindly provided by G. Wong and J. Morris, Genetics Institute). The preparation was estimated to be 50% pure by sodium dodecyl sulfate (SDS) gel electrophoresis (the major contaminant remaining was albumin). The sample was found to support half-maximal colony formation in the murine macrophage colony assay at a final dilution of 1:300,000.

Erythropoietin. Human urinary ep was obtained from Terry Fox Laboratories, Vancouver, British Columbia, Canada. Human recombinant ep was obtained from transfected Simian COS-1 or CHO cells (generously provided by A. Mufson and T. Gesner, Genetics Institute). The specific activities of the human urinary ep and the recombinant preparations were 150 U/mL and 300,000 U/mg respectively. Earlier studies showed that there was no difference in the responsiveness of human progenitors to human urinary or recombinant preparations.

Progenitor purification. Normal human bone marrow was obtained by aspiration from healthy adult volunteers. The procedure and attendant risks were discussed with all donors before bone marrow aspiration in accordance with institutional guidelines, and all gave informed consent. The marrow suspension was separated over Ficoll-Paque (1.077 g/cm³, Pharmacia Fine Chemicals, Piscataway, NJ) at 400 g for 40 minutes at 20°C, and the interface
mononuclear cells were collected, washed three times and resuspended in Iscove's modified Dulbecco's medium (IMDM) that contained 20% fetal calf serum (FCS). The bone marrow cells were incubated either overnight or for one hour at 37°C/5% CO2 and nonadherent cells removed. The progenitor cells in the nonadherent cell fractions were enriched by immune absorption to immunoglobulin-coated magnetic beads (Dynabeads, M-450, P and S Biochemical Inc., Gaithersburg, MD). The beads were coated with rabbit anti-mouse immunoglobulins (Zymed Laboratories, San Francisco, CA), according to the instructions issued by the manufacturers. The bone marrow cells were labeled at 4°C for 30 minutes with optimal concentrations of a panel of eight monoclonal antibodies directed against myeloid, lymphoid, and erythroid maturation antigens, as previously described.9,12 The cells were washed twice in IMDM and once in phosphate-buffered saline (PBS) containing 5% FCS. Immunomagnetic beads were added to the cell pellet at approximately 5 beads/cell. The mixture was resuspended and incubated for 30 minutes at 4°C. Cells that had bound to the beads were then separated using a magnet. The supernatant containing the antibody negative (ab-) cells was collected, centrifuged, and resuspended in IMDM without FCS.

Culture Procedures

Serum-containing Cultures. Bone marrow cells were cultured in a mixture containing 30% FCS, 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), 10^-4 M beta mercaptoethanol (Sigma Chemical Co.), penicillin, streptomycin, and 0.9% methylcellulose.

Serum-deprived cultures. Cells were cultured in a mixture containing 1% deionized BSA (Cohn fraction V, Sigma Chemical Co.), 2 x 10^-4 M BSA absorbed cholesterol (Sigma Chemical Co.), 300 μg/mL iron-saturated transferrin (Boehringer-Mannheim, Indianapolis, IN), 10 μg/mL insulin (Sigma Chemical Co.), 10^-4 M beta mercaptoethanol, and IMDM (Hazelton, Lenoxa, KS). Since the BSA may have been contaminated with trace levels of serum proteins, we refer to this medium as serum-deprived. Varying concentrations of the recombinant CSFs or serum-free Mo lymphoblast cell line-conditioned medium (Mo-CM) (provided by Drs. D. Golde and J. Gasson, University of California at Los Angeles School of Medicine) as positive controls were added to the cultures on day 0 or day 3. We chose to use Mo-CM to provide a standard of growth to which the serum-deprived cultures with and without growth factors could be compared. The amounts of the lineage specific growth factors added on day 3 were optimal concentrations determined from preliminary dose response experiments. The final cell concentration was 5 x 10^5/mL and either 1 or 0.5 mL duplicates were plated in 35 mm petri dishes or in flat bottom 24 well tissue culture plates (Flow Laboratories, Inc., McLean, VA), respectively, and incubated at 37°C at a high humidity 4% CO2/96% air incubator. Erythroid colony-forming units (CFU-E) were counted on day 7 and BFU-E, CFU-G, CFU-M, and CFU-GM were counted on day 14. Colonies of a panel of eight monoclonal antibodies directed to stimulatory CSF were considered "significant." Because of the number of P-values generated for these analyses and the reuse of the data, P < 0.005 will be considered to be "significant."

RESULTS

Comparison of Colony Formation in Serum-replete and Serum-deprived Conditions

To evaluate the capacity of serum-deprived cultures to support erythroid and myeloid colony growth, we performed preliminary experiments in which we compared the number of colonies that form in serum-replete and serum-deprived conditions. The results were as follows.

Low density, nonadherent, and ab- progenitor-enriched bone marrow cells were cultured with Mo-CM and ep. Progenitor recovery in the ab- fraction was 22% to 200% respectively for three experiments. There was no selective loss of progenitor recovery in serum-deprived versus serum-replete culture conditions. The proportion of erythroid progenitors recovered in serum-deprived cultures ranged from 0.4 to 2.4 for CFU-E and 0.9 to 2.4 for BFU-E when compared with serum-containing cultures for both the nonadherent and progenitor-enriched cell fractions. The superior BFU-E and CFU-E growth in some serum-deprived cultures may reflect difficulties in standardizing FCS batches. Although the FCS was screened for erythroid colony support, it is not as good as our current batch of "defined FCS." Myeloid colony growth in serum-deprived cultures was usually not as good as in serum-replete cultures: the proportion of granulocyte and monocyte colonies observed in serum-deprived cultures ranged from 0.1 to 1.3 times the number observed in serum-replete conditions.

IL-3 and GM-CSF Alone Are Inadequate Stimuli of CFU-G

We next used progenitor-enriched bone marrow cells from three donors to evaluate the ability of IL-3 and GM-CSF to support colony formation in serum-deprived conditions. Surprisingly, both IL-3 and GM-CSF were poor stimuli of CFU-G (Fig 1A, B, IL-3 and GM-CSF alone), since at maximal concentrations (extrapolated from serum-replete cultures) only 28% and 17% of the colonies produced by optimal concentrations of Mo-CM were observed. Although G-CSF alone (day 0 addition) was an adequate stimulus for CFU-G derived colony formation in serum-deprived conditions and induced 87% of the colonies observed in Mo-CM (not shown), when G-CSF was added alone on day 3, only 11% of the CFU-G obtained with Mo-CM were recovered (Fig 1A). Conditioned medium from mock G-CSF transfecunt had no colony stimulating activity, and did not enhance or inhibit growth induced by other CSFs. When IL-3 was added on day 0 and G-CSF added on day 3, 84% of CFU-G were recovered (Fig 1A, P < 0.001). The substitution of GM-CSF for IL-3 resulted in CFU-G recovery of 114% (Fig 1B, P = 0.002). If M-CSF
cultures of enriched progenitor cells, very few CFU-M derived colonies were observed. When IL-3 was added on day 0 and M-CSF on day 3, up to 85% of the CFU-M that were induced with Mo-CM were recovered (Fig 2A). When GM-CSF was substituted for IL-3 and tested in combination with M-CSF, there was not a statistically significant difference in the number of CFU-M derived colonies compared with GM-CSF alone (Fig 2B, P = 0.23).

**IL-3 Is a More Potent Stimulus of BFU-E than GM-CSF**

When ep alone was added to the cultures on day 3, almost no BFU-E derived colonies were observed. When IL-3 was added on day 0 and ep on day 3, almost full BFU-E recovery was obtained in comparison to Mo-CM and ep (Fig 3). However, when GM-CSF was added on day 0 and ep on day 3, a mean of approximately one third of BFU-E were recovered (IL-3 v GM-CSF, P = 0.001).

**IL-3 but Not GM-CSF Is an Inadequate Stimulus of CFU-M**

We next tested the ability of IL-3 to support the development of CFU-M. IL-3 induced only 24% of the CFU-M observed in cultures that contained Mo-CM (Fig 2A). In contrast, GM-CSF alone was able to induce the formation of similar numbers of colonies to Mo-CM (Fig 2B).

When M-CSF was added alone either on day 0 (not shown) or day 3 at optimal concentrations to serum-deprived cultures of enriched progenitor cells, very few CFU-M derived colonies were observed. When IL-3 was added on day 0 and M-CSF on day 3, up to 85% of the CFU-M that were induced with Mo-CM were recovered (Fig 2A). When GM-CSF was substituted for IL-3 and tested in combination with M-CSF, there was not a statistically significant difference in the number of CFU-M derived colonies compared with GM-CSF alone (Fig 2B, P = 0.23).

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The Combination of IL-3 and GM-CSF Is a More Effective Stimulus of BFU-E than Either Factor Alone

In order to determine whether combinations of IL-3 and GM-CSF were additive or synergistic, cultures were established in IL-3, GM-CSF, or both IL-3 and GM-CSF. On day 3, either ep, G-CSF, or M-CSF was added to the culture dishes, and the cultures scored 11 days later for BFU-E, CFU-G, or CFU-M respectively. Fig 4 shows that the combination of IL-3 and GM-CSF was a more potent stimulus of BFU-E and CFU-M than either factor alone. The interaction was significant for BFU-E, indicating synergy (P < 0.001) but did not reach significance for CFU-M (P = 0.012). The CFU-G cultures did not show evidence of synergy.

DISCUSSION

Despite the availability of recombinant CSFs and techniques to enrich for progenitor cells, results with “standard” 30% FCS containing culture systems may not reflect the biologic activities of the CSFs. Better defined culture conditions may provide insight into the actions of the CSFs. Using serum-deprived conditions, we show that both IL-3 and, to a lesser extent, GM-CSF, are poor stimuli for colony formation; very few CFU-G derived colonies form in response to either factor alone. Similarly, CFU-M were not observed in the presence of IL-3, although CFU-M derived colonies were observed in response to GM-CSF alone. When IL-3 stimulated cultures were supplemented with either G-CSF, M-CSF, or ep, almost full recovery of granulocyte, monocyte, and erythroid colonies, respectively, was noted. When GM-CSF stimulated cultures were supplemented with G-CSF, CFU-G recovery was markedly increased; in the presence of ep, however, approximately one third of BFU-E were recovered in comparison with IL-3/ep stimulated cultures. The BFU-E and CFU-G results are consistent with two recent reports: in contrast to our results, however, Sonoda et al found that M-CSF did not support colony growth when combined with IL-3 or GM-CSF. Finally, the combination of IL-3 and GM-CSF was synergistic for BFU-E and showed an additive effect on CFU-M.

Migliaccio et al recently reported that the combination of IL-3 (20 pM), GM-CSF (450 pM), and ep (1.5 U) in serum-deprived cultures induced equivalent numbers of BFU-E derived colonies to those observed in serum-replete
cultures that contained PHA-LCM and ep. They did not report whether synergistic effects of IL-3 and GM-CSF were present at higher CSF concentrations. While we were not able to recover all the BFU-E capable of colony formation in serum-replete PHA-LCM, synergistic effects of IL-3 and GM-CSF were observed at concentrations as high as 5 nmol/L. BFU-E numbers were slightly increased at 5 nmol/L IL-3 and GM-CSF in comparison with cultures established at 0.5 nmol/L. Colonies were much larger, however, and averaged 2 x 10^3 erythroblasts per colony at 5 nmol/L CSFs in comparison with 10^2 cells per colony at the lower concentrations. In contrast with our results, Migliaccio et al. also reported that IL-3, GM-CSF, and G-CSF induced more CFU-G than either IL-3 and G-CSF or GM-CSF and G-CSF.

These serum-deprived results are partly consistent with in vivo effects, since recent primate studies show that infusions of IL-3 or low doses of GM-CSF alone do not significantly increase granulocyte, monocyte, or reticulocyte counts. When IL-3 was given to simians for seven days followed by a four-day infusion of low dose GM-CSF, marked hematopoietic stimulation was observed, with a consistent increase in polymorphonuclear neutrophils, monocytes, reticulocytes, and platelets. GM-CSF alone does increase granulocyte and monocyte counts when given in vivo to monkeys or humans by continuous infusion, and our results may only have predicted a monocyte increase. However, hematopoiesis in vivo occurs in a plasma environment, and although levels of M-CSF in human serum are detectable, the levels of G-CSF are unknown. Until circulating levels of all the CSFs can be assayed precisely, it may be difficult to evaluate the most optimal culture system that would reflect or predict in vivo results.

The experiments reported here show interactions of IL-3 and GM-CSF with each other and with lineage-specific factors. These interactions concern a subset of progenitors, since colony numbers in serum and certain batches of serum-replete PHA-LCM were higher for all progenitor classes. It was the ability to specifically stimulate this subset of progenitors alone that allowed these interactions to be discerned, since culture established in serum-replete conditions did not reveal synergism between IL-3 and GM-CSF (not shown). However, it should be borne in mind that unidentified serum factors may be important for the proliferation of some progenitor classes.

Our previous results with serum-replete cultures suggested that IL-3 was a more potent stimulus of BFU-E than GM-CSF. Although the recombinant GM-CSF used in these earlier experiments was highly purified, the IL-3 was, however, obtained from medium conditioned by IL-3-transfected COS cells that transiently expressed high levels of the protein. In the experiments reported here, we used highly purified preparations of recombinant E. coli IL-3 and CHO cell GM-CSF. The results in serum-free culture confirm our earlier data, in that almost full recovery of BFU-E was obtained in IL-3/ep, whereas significantly fewer BFU-E were recovered in GM-CSF/ep.

In view of the recent use of the CSFs in therapeutic trials, these results may be important in two ways. First, they suggest that hematopoietic responses may best be obtained by combining an early-acting factor such as GM-CSF or IL-3 with one that acts later during differentiation. Second, it may be possible to optimally induce granulocyte, monocyte, or erythroid differentiation by selecting the appropriate two CSFs. In vivo primate studies are in progress to evaluate these predictions.

REFERENCES
HUMAN CSFs IN SERUM-DEPRIVED CULTURES


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