Stimulating Spectrum of Human Recombinant Multi-CSF (IL-3) on Human Marrow Precursors: Importance of Accessory Cells

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Recently, human multi-CSF was obtained by molecular cloning. In the present study, the effects of multi-CSF in vitro were investigated by comparative culture of whole bone marrow or progenitor cells obtained by sorting the cell fraction that binds the monoclonal antibody (MoAb) B13C5 (CD 34). Multi-CSF stimulated erythroid (BFU-E), multipotent (CFU-GEMM) and eosinophil (CFU-Eo) colonies in cultures of the progenitor cell enriched fraction, whereas (besides BFU-E, CFU-GEMM, and CFU-Eo) granulocyte (CFU-G), granulocyte-macrophage (CFU-GM), and macrophage (CFU-M) colony-forming cells also were stimulated by multi-CSF when unfraccionated bone marrow was cultured. Reconstitution of the progenitor cell fraction (B13C5 positive) with the B13C5-negative population restored the broad spectrum of progenitor cell stimulation. This suggested that accessory cells are required for expression of the full spectrum of progenitor cell stimulation by multi-CSF. Subsequently, specific marrow cell populations, including T lymphocytes, granulocytic cells, and monocytes, were prepared by using selected MoAbs in complement-mediated lysis or cell sorting, added to cultures of hematopoietic progenitors and tested for accessory cell function. The results demonstrate that small numbers of monocytes permit the stimulation of CFU-G, CFU-GM, and CFU-M by multi-CSF. These monocyte-dependent stimulating effects on CFU-G, CFU-GM, and CFU-M could also be achieved by adding recombinant GM-CSF as a substitute for monocytes to the cultures. Therefore, multi-CSF most likely has direct stimulative effects on BFU-E, CFU-GEMM, and CFU-Eo and indirect effects on CFU-G, CFU-GM, and CFU-M in the presence of monocytes.

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numerical proportions of the two cell populations in the Ficoll-
fractionated marrow specimen unless stated otherwise.

Complement-mediated cytolysis. In several experiments, we
used a different approach for the addition of subfractions from the
B13C5-negative cell fraction. After sorting, the B13C5-negative cells
were incubated with either MoAb VIM-2 or T3 at optimal concen-
trations (30 minutes on ice) and then incubated with rabbit comple-
ment at a final concentration of 40% (30 minutes at 25°C) and
washed twice. These VIM-2- or T3-depleted B13C5-negative cells
were then added to the appropriate cultures and tested for accessory
abilities.

CFU-GEMM culture assay. Mixed colonies were grown as
described before by Fauser and Messner with slight modifica-
tions. Sorted or unsorted marrow cells were cultured in a 1-mL
mixture of Iscove's modified Dulbecco's medium (IMDM), 1.1%
methylcellulose, 30% autologous heparinized plasma, BSA, transferrin,
lecithin, sodium-selenite and 2-mercaptoethanol. Cells were added at a concentration of 8 \times 10^7/mL in total marrow cultures
and 0.2 to 0.5 \times 10^7/mL for the B13C5-positive cell fractions.
Exogenous growth stimuli were added in the form of various
concentrations of multi-CSF and recombinant human erythropoietin
(Epo) at a concentration of 1 U/mL (Kirin-Amgen, Thousand Oaks,
CA). Cultures with 10% of a medium stimulated by leukocytes in
the presence of 1% phytohemagglutinin (PHA-LCM), as based on
the original CFU-GEMM assay, were done for comparison. Dishes were
incubated at 37°C and 100% humidity in an environment of 5% CO2
in air. Colonies were scored at day 15 and identified by their distinct
morphological appearance at 100x magnification. Numbers of
colonies refer to the means of duplicate cultures. In selected cases,
the nature of the colonies verified cytologically after they had been
plucked from the plates with a finely drawn Pasteur pipette,
and stained with May-Grünwald-Giemsa. Mixed colonies were
always verified cytologically. Megakaryocyte colonies were not
assayed since CFU-Meg could not be detected reproducibly in every
bone marrow sample. However, ~15% of CFU-GEMM in the
multi-CSF-stimulated cultures contained megakaryocytes.

RESULTS

Multi-CSF as a stimulator of purified hematopoietic
progenitor cells. To minimize a possible interference of
nonclonogenic accessory cells, we enriched progenitor cells
from normal human bone marrow by sorting the cell fraction
positive for MoAb B13C5 (CD 34), and cultured the cells at
low cell concentrations (2 to 5 \times 10^7/mL) in the presence of
multi-CSF. The results of a representative experiment are
shown in Fig 1. When Epo was added as the only exogenous
growth-stimulating factor some background erythroid bursts
(CFU-E) were formed. BFU-E numbers rose markedly when
graded concentrations of multi-CSF were added and reached a
plateau at 1 to 3% (vol/vol) multi-CSF. In subsequent
experiments, a concentration of 3% multi-CSF was used.
Eosinophil (CFU-Eo) and multipotent (CFU-GEMM) colonies
appeared in multi-CSF-stimulated cultures, and their numbers rose
as a function of increasing concentrations of multi-CSF. When only multi-CSF but no Epo was added to the
cultures, neither red nor mixed colonies appeared,
whereas the number of eosinophil colonies remained con-
stant. A remarkable number of eosinophil colonies was
formed in the presence of multi-CSF with or without Epo.
Multi-CSF did not stimulate significant numbers of granulo-
cyte (CFU-G), granulocyte-macrophage (CFU-GM),
or macrophage (CFU-M) colonies. The numbers of BFU-E
obtained with multi-CSF were similar to those stimulated by
a crude conditioned medium (PHA-LCM), but somewhat
lower numbers of CFU-GEMM and CFU-Eo were obtained
with multi-CSF than with PHA-LCM. In several experi-
ments with a greater concentration of multi-CSF (10% vol/vol), the colony numbers, in particular those of BFU-E,
declined. This reduction suggests the presence of inhibitory
factors in the multi-CSF preparation (COS supernatant).
Recently, we used partially purified Escherichia coli-derived
multi-CSF in concentrations up to 30 times (expressed as
activity) greater than the maximal concentration of COS-
supernatant multi-CSF, and demonstrated no inhibitory
effect. The pattern of stimulation, ie, induction of colony
formation from BFU-E, CFU-Eo, and CFU-GEMM, was
identical for both types of multi-CSF.

Role of accessory cells. To examine a possible role of
B13C5-negative accessory cells in the stimulatory effects of
multi-CSF, we compared the effects of multi-CSF on: (a) a
mock-sorted cell fraction, ie, passed through the cell sorter
without selecting for a specific marker (total marrow
nucleated cells) (Fig 2A); (b) the sorted B13C5-positive cell
population (precursor cell fraction) (Fig 2B); and (c) the
B13C5-positive cell fraction supplemented with the number
of B13C5-negative cells that reconstituted the total marrow
cell population (Fig 2C). Multi-CSF appeared to stimulate
not only BFU-E, CFU-GEMM, and CFU-Eo but also
CFU-G, CFU-GM and CFU-M in unfractonated bone
marrow cells (Fig 2A). This contrasts with the pattern of

Fig 1. Colony formation in response to multi-
CSF: culture of purified human hematopoietic pro-
genitors. Numbers of erythroid, multipotential,
and four classes of myeloid colonies (Eo, G, GM, M) in vitro are plotted as a function of
increasing concentrations of recombinant multi-
CSF. The results from one representative experi-
ment are shown. The B13C5 (CD34)-positive exper-
tment was obtained by cell sorting from 8 \times 10^6
mononuclear marrow cells and represented 5% of
the original cell population. Values represent mean
colony counts of duplicate cultures. Epo (1 U/mL)
was added to all cultures; in cultures without Epo
and without multi-CSF no colonies appeared. Col-
ony growth after stimulation with 10% PHA-LCM
is shown for comparison. Data for one of duplicate
experiments are shown.
stimulation in the B13C5-positive precursor cell fraction (Figs 1 and 2B): ie, stimulation of BFU-E, CFU-GEMM, and CFU-Eo only, but not the other myeloid colony-forming cells (CFU-G, CFU-GM, CFU-M) (Fig 2C). The addition of B13C5-negative cells to the B13C5-positive cell fraction completely restored the stimulative effect of multi-CSF on BFU-E, CFU-GEMM, and CFU-M. The B13C5-negative cell fraction per se did not contain significant numbers of colony-forming cells (Fig 2D). Because these experimental data suggested that CFU-G, CFU-GM, and CFU-M colony formation in response to multi-CSF depended on the presence of B13C5-negative cells or a subpopulation of these in culture, we subsequently examined the effect of the removal of specific subpopulations from this cell fraction using complement-mediated cytolysis of VIM-2 (myelomonocytic) or T3 (mature T lymphocytes) positive cells (Fig 3). VIM-2 lysis abrogated the augmentory effect of the B13C5 negative cells whereas T3 lysis did not. These results suggested that
the active accessory cells are VIM-2 positive and thus belong to the myelomonocytic lineage.

Following an alternative approach, specific VIM-2, T3 (mature T lymphocytes), B44.1 (monocytes), and B4.3 (granulocytes) positive subsets were obtained from the B13C5-negative fraction by cell sorting (Fig 4) and examined for their abilities to exert the accessory cell effects. Supplementation of VIM-2-positive or B44.1-positive cells to the B13C5-positive progenitor cell fraction restored the development of colonies originating from CFU-G, CFU-GM and CFU-M in cultures stimulated with multi-CSF. As few as 2.5 x 10^4 VIM-2 or 0.5 x 10^4 B44.1 surface marker-positive cells were capable of enhancing colony growth. However, 2 x 10^4 B4.3-positive granulocytes or T3-positive lymphocytes were ineffective. Indeed, CFU-GEMM, BFU-E, and CFU-Eo were also slightly susceptible to the potentiating effect of monocytes, at least when large numbers of monocytes were added (Fig 4G).

We examined whether the effect of the addition of monocytes to cultures of the B13C5-positive progenitor cell fraction to evoke CFU-G, CFU-GM, and CFU-M colony formation could be mimicked by supplementing exogenous GM-CSF or G-CGF (instead of monocytes) to the multi-CSF cultures (Fig 5). When G-CSF or GM-CSF were added to multi-CSF cultures of purified bone marrow progenitors, additional CFU-G or CFU-G, CFU-GM and CFU-M were induced to colony formation. There was no evidence for a synergistic effect between these factors. When all three factors (ie, multi-CSF, GM-CSF, and G-CSF) were included in culture, no significant further increase in colony formation was seen above the level of multi-CSF plus GM-CSF stimulation. This indicates that multi-CSF and GM-CSF in conjunction provide optimal stimulation of CFU-G, CFU-GM, and CFU-M from the B13C5-positive progenitor cell fraction and that GM-CSF can substitute the monocyte accessory cell effect.

**DISCUSSION**

We assessed the stimulating abilities of the recombinant human growth factor multi-CSF. In cultures of enriched human hematopoietic progenitor cells (based on B13C5 reactivity), multi-CSF stimulated BFU-E, CFU-Eo, and CFU-GEMM. In the presence of B13C5-negative cells, the spectrum of multi-CSF stimulation was broader and also included CFU-G, CFU-GM, and CFU-M. These data suggest a direct stimulative effect of multi-CSF on BFU-E, CFU-Eo, and CFU-GEMM and an effect on CFU-G, CFU-GM, and CFU-M in the presence of a secondary cell. Experiments based on the addition of specific subsets of cells (selected by cell sorting) as well as elimination of these subsets from the accessory cell fraction (by complement-mediated lysis) identified a VIM-2 and B44.1 surface marker-positive cell population as the active accessory cell. The accessory cell function could not be attributed to the subsets that expressed the T-lymphocytic T3 or granulocytic B4.3 markers. The VIM-2 and B44.1-positive surface phenotype of the accessory cells demonstrates the monocytic identity of the cells. It became apparent that the accessory cell phenomenon was cell dose dependent and that minimal numbers (0.5 x 10^4 per dish) of these monocytic cells were sufficient to allow the outgrowth of CFU-G, CFU-GM, and CFU-M in multi-CSF-stimulated cultures. The addition of four times the proportional number of VIM-2-positive cells or B44.1-positive cells to purified progenitors further elevated GM and M colony numbers, and also raised the number of BFU-E.

![Fig 4. Colony formation in response to multi-CSF: effect of addition of different subpopulations of accessory cells. Purified hematopoietic progenitor cells (B13C5 positive; 4 x 10^4 cells/dish) were plated in with 3% multi-CSF and Epo. To these cultures VIM-2-positive, B44.1-positive, B4.3-positive, or T3-positive cells were added and examined for the colony formation.]
CFU-Eo, and CFU-GEMM. Three possible mechanisms through which monocytes enhance growth of these colony types stimulated by multi-CSF can be proposed: (a) production of growth factors (e.g., GM-CSF or M-CSF) by monocytes in response to multi-CSF, (b) production of growth factors by monocytes independent of multi-CSF, and (c) cell–cell interactions between monocytes and colony-forming cells. The present experiments do not allow a distinction between the alternative mechanisms for the enhancement of CFU-G, CFU-GM, and CFU-M colony growth. The possibility that monocytes produce growth factors that can induce CFU-G, CFU-GM, and CFU-M would at least be consistent with the observation that monocytes can be stimulated to produce colony-stimulating factors. More recently, specific evidence was obtained demonstrating that monocytes may produce M-CSF, interferon (IFN), tumor necrosis factor (TNF), and GM-CSF. This could explain our results showing that the addition of recombinant GM-CSF could fully substitute the monocyte effect and that G-CSF could partly replace the role of the monocytes in the multi-CSF cultures, thereby resulting in the induction of G, GM, and M colony types from purified marrow progenitor cells.

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