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Synergistic suppression: Anomalous inhibition of the proliferation of factor-dependent hemopoietic cells by combination of two colony-stimulating factors

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ABSTRACT **Cells of the continuous murine hemopoietic cell line FDC-P1 expressing macrophage-colony-stimulating factor (M-CSF) receptors following retroviral insertion of murine c-*fms* cDNA proliferated clonally when stimulated by granulocyte/macrophage (GM)-CSF, multipotential CSF, or M-CSF. However, M-CSF combined with either GM-CSF or multi-CSF, even at low CSF concentrations, strongly inhibited colony formation, with loss of clonogenicity in affected cells accompanied by increased macrophage differentiation. Stimulation by these CSF combinations did not induce short-term changes in CSF receptor expression or internalization. FDC-P1 cells expressing another inserted tyrosine kinase receptor, basic fibroblast growth factor receptor, did not exhibit suppression when GM-CSF was combined with fibroblast growth factor. This phenomenon of synergistic suppression may have relevance for the future clinical use of combinations of CSFs, because a potentially similar suppression is also observable with some normal macrophage progenitor cells.**

When used individually, each of the four glycoprotein colony-stimulating factors GM-CSF, G-CSF, M-CSF, and multi-CSF (interleukin 3) is a highly active proliferative stimulus for granulocyte/macrophage precursors (1). In general, combination of two or more of these CSFs in semisolid cultures leads to enhanced proliferative responses (2). However, in cultures of normal mouse marrow cells, it was noted that combination of GM-CSF with M-CSF led to a partial suppression of macrophage colony formation (2, 3).

Cells of the continuous murine hemopoietic line FDC-P1 respond to proliferative stimulation either by GM-CSF or multi-CSF. With a variant of this line also expressing the c-*fms* product, the M-CSF receptor, it was noted that extended stimulation by GM-CSF suppressed the expression of M-CSF receptors (3). In cultures of FDC-P1 cells that had been induced to display receptors for M-CSF by use of a c-*fms*-expressing retrovirus, it was observed that combination of M-CSF with either GM-CSF or multi-CSF led to suppression of the number and size of colonies developing. The present experiments were undertaken to explore the nature of this suppressive action.

MATERIALS AND METHODS

FDC-P1 and FD-c-fms Cell Lines. Cells of the FDC-P1 line (obtained originally from T. M. Dexter, Paterson Institute, Manchester) are dependent for survival and proliferation on continuous stimulation by either GM-CSF or multi-CSF (4). The cells were maintained in suspension culture in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and were subcultured when they reached a density of 10^6 cells per ml. Derivative FD-c-fms cell lines were generated by cocultivating FDC-P1 cells for 48 hr, in the presence of pokeweed mitogen-stimulated spleen cell-conditioned medium as a source of multi-CSF (2), with a ψ-2 cell line producing infective Zen retrovirus containing a murine c-*fms* cDNA insert (4). After cocultivation, the cells were washed three times and then cultured at a density of 300 cells per ml in semisolid cultures stimulated by recombinant murine M-CSF at 1000 units/ml. After 7 days of incubation, individual colonies were removed with a fine pipette and expanded as cloned FD-c-fms cell lines in suspension cultures containing M-CSF at 1000 units/ml. The derivation of the FDC-P1 cell line expressing fibroblast growth factor (FGF) receptors, FD-FGFR-IL.B, has been described (5).

Clonal Cultures. Semisolid cultures were set up in 1-ml volumes in 35-mm plastic Petri dishes. FDC-P1 or FD-c-fms cells were washed three times to remove the CSF used to maintain the suspension cultures and then were cultured in Dulbecco's modified Eagle's medium with a final concentration of 20% newborn calf serum and 0.3% agar (Bacto-Agar, Difco) (2). In some experiments, the gels were prepared using 1.5% methylcellulose (Fluka) or 0.25% purified agarose (SeaPlaque; FMC). Cultures were stimulated by addition of CSF in a volume of 0.1 ml prior to the addition of the cell suspension in the semisolid medium. After gelling, cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Colony numbers were counted at ×35 magnification with a dissection microscope. Mean colony cell counts were determined by pooling 50 sequentially harvested colonies in 1 ml of 5% serum in 0.9% NaCl, then resuspending the colony cells with a Pasteur pipette and performing cell counts with a hemocytometer. Recloning of single colony or cluster cells was performed by micromanipulation (2).

Flow Cytometry. FDC-P1 or FD-c-fms cells in suspension culture were labeled by hybridoma supernatants containing the rat monoclonal antibody Mac1, Mac2, Mac3, or GR-1, followed by phycoerythrin-conjugated anti-rat monoclonal antibody. Cells were then analyzed with a FACScan instrument (Beckman). Cell volume estimates were performed using a Coulter Counter.

Stimuli. The CSFs used were recombinant murine preparations purified after expression in *Escherichia coli* or yeast. The specific activities were as follows: M-CSF, 10^8 units/mg; GM-CSF, 3×10^8 units/mg; multi-CSF, 10^8 units/mg. Fifty units is defined as the concentration of a CSF stimulating the formation of 50% maximal colony numbers in 1-ml agar cultures of 75,000 C57BL bone marrow cells. Bovine basic FGF was purchased from Boehringer Mannheim.

Binding and Internalization Studies. Purified recombinant yeast-derived M-CSF and *E. coli*-derived GM-CSF were iodinated by a modified iodine monochloride method, and

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Abbreviations: CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; M-CSF, macrophage CSF; multi-CSF, multipotential CSF (interleukin 3); FGF, fibroblast growth factor.
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internalization experiments were performed at 37°C and analyzed as described (6). Receptor interactions were determined by preincubating cells with one CSF for 60 min at 37°C, washing them, and then performing binding experiments with another CSF for 3 hr at 4°C.

RESULTS

Suppression of FD-c-fms Clonogenic Cells. In agar cultures using 300 FD-c-fms cells from six cloned cell lines, at least five of which were shown by Southern analysis of viral insertion sites to have been independently derived, it was observed that all responded to stimulation by GM-CSF (1000 units/ml) and to a lesser degree to stimulation by M-CSF (1000 units/ml) (Fig. 1). Combination of the two stimuli resulted in a marked reduction in the number and size of colonies forming compared with colonies stimulated by GM-CSF alone. Colonies stimulated by GM-CSF were tight spherical aggregates of up to 15,000 cells. Colonies stimulated by M-CSF, although smaller, were also compact. Colonies formed after stimulation by the combination of GM-CSF and M-CSF were very small and were composed of dispersed cells of irregular size mixed with some dying cells. Combination of multi-CSF with M-CSF also reduced the number and size of colonies developing, but the reduction was not as large as that observed with the combination of GM-CSF and M-CSF (Fig. 2). Combination of GM-CSF with multi-CSF did not reduce colony numbers and usually resulted in colonies similar in size to those stimulated by either CSF alone.

A difference in the morphology of clones developing after combined stimulation by GM-CSF plus M-CSF was evident within 24–30 hr of initiation of the cultures. At this time, these clones tended to be composed of dispersed cells that were often of opaque appearance and irregular shape. After 30 hr of incubation, reculture of micromanipulated single cells from such clones (two to eight cells in size) in recipient cultures containing only GM-CSF revealed a significant reduction in the frequency of clonogenic cells (Table 1). However, most clonogenic cells that were present generated typical compact colonies of normal size in secondary recipient cultures stimulated by GM-CSF. The small 7-day colonies stimulated by GM-CSF plus M-CSF contained greatly reduced numbers of clonogenic cells (25 ± 28 per colony) when assayed in secondary cultures stimulated by GM-CSF

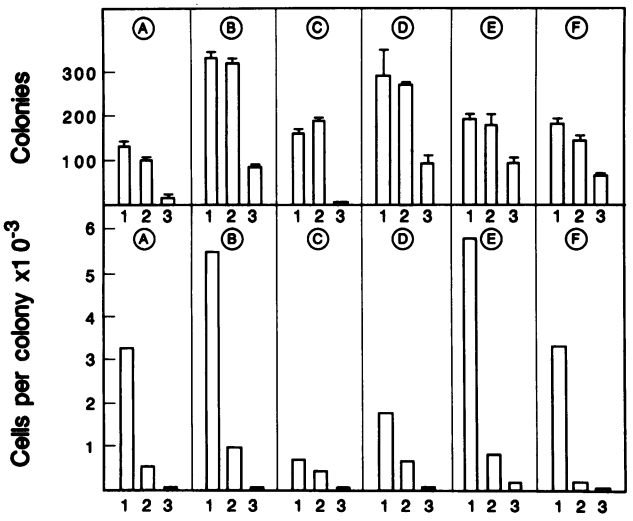

FIG. 1. Six FD-c-fms cell lines (*A–F*) showing anomolous inhibition of colony formation or colony cell numbers when stimulated by a combination of M-CSF and GM-CSF (each at 1000 units/ml) (bars 3) versus stimulation by GM-CSF (bars 1) or M-CSF (bars 2) alone.

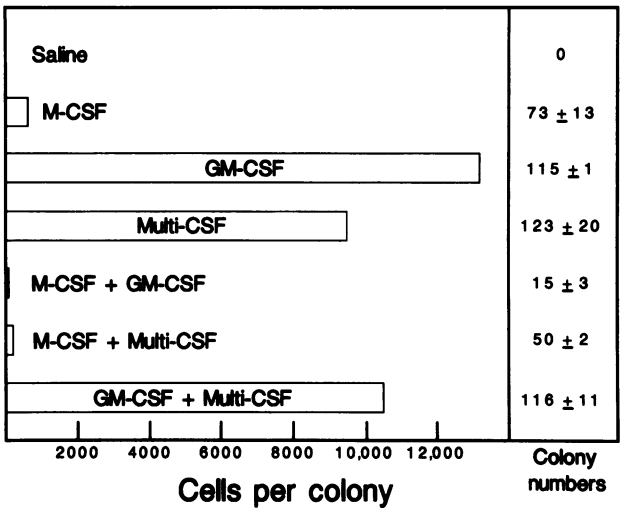

FIG. 2. Combination of M-CSF (1000 units/ml) with either GM-CSF or multi-CSF (1000 units/ml) reduced the number and size of colonies formed by 150 FD-c-fms cells compared with those stimulated by each stimulus alone. Data are means ± standard deviations from four replicate cultures.

compared with the number (1710 ± 630 per colony) in 7-day colonies stimulated by GM-CSF alone.

The suppression of colony formation by FD-c-fms cells by the combination of GM-CSF with M-CSF was not dependent on cell or colony crowding. Within the range of 12 to 300 cells cultured per ml, the number of colonies forming was linear with respect to cultured cell numbers in cultures stimulated by GM-CSF, M-CSF, or a combination of both CSFs. Furthermore, the degree of reduction of colony size induced by combination of GM-CSF with M-CSF was not influenced by the number of cells cultured in this concentration range (data not shown).

Influence of CSF Concentration on Clonal Suppression. In cultures of 100 FD-c-fms cells, addition of equal concentrations of GM-CSF and M-CSF in the concentration range from 2 to 1000 units/ml (Fig. 3) showed that unambiguous inhibition by the combination first became detectable with a concentration of 60 units of each per ml, and suppression became progressively more apparent with increasing CSF concentrations. This inhibition was not simply the nonspecific consequence of a higher final concentration of CSF in the cultures, since colony size increased progressively with increasing concentrations of either CSF when used alone. In

Table 1. Reculture of single cells from clones of FD-c-fms cells stimulated for 30 hr by GM-CSF or by GM-CSF plus M-CSF

Stimulus initiating clones	No. of cells transferred	No. producing		
		Colony	Cluster	0 or 1 cell
Experiment 1				
GM-CSF	34	31	1	2
GM-CSF + M-CSF	49	22	7	20
Experiment 2				
GM-CSF	39	37	0	2
GM-CSF + M-CSF	35	23	4	8
Experiment 3				
GM-CSF	38	29	1	8
GM-CSF + M-CSF	52	18	7	27

Three experiments in which cultures of 100 FD-c-fms cells from two different lines were stimulated by GM-CSF (1000 units/ml) alone or with M-CSF (1000 units/ml). After 30 hr of incubation, single cells from developing clones were transferred by micromanipulation to recipient cultures containing GM-CSF (1000 units/ml). Secondary cultures were scored after 7 days of incubation.

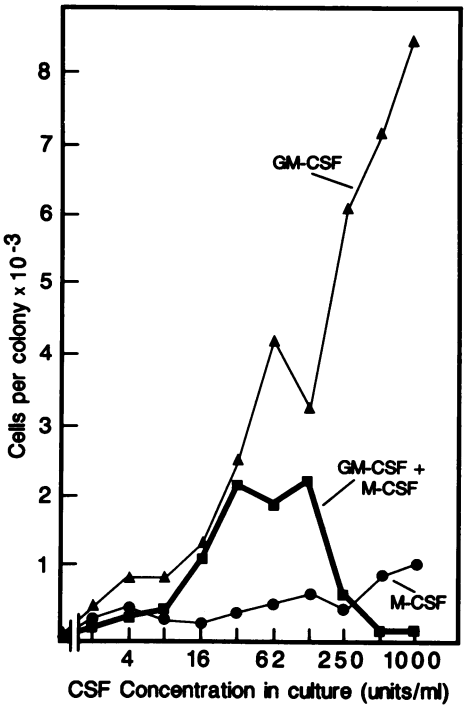

FIG. 3. Inhibition of FD-c-fms colony cell numbers became detectable with a combination of 60 units of GM-CSF plus 60 units of M-CSF per ml and became more pronounced as concentrations were increased beyond this point.

cultures prepared using one CSF at 1000 units/ml and increasing concentrations of the second CSF, inhibition was first observed with a lower concentration (≈16 units/ml) of the second CSF than in cultures containing equal concentrations of both CSFs.

Influence of the Suspension Medium. FD-c-fms colonies did not grow well in 0.3%, 0.25%, or 0.2% agarose, and the cloning efficiency of cells when stimulated by GM-CSF was significantly lower than in parallel agar cultures. Despite this, combination of GM-CSF with M-CSF in 0.25% agarose cultures reduced colony numbers (Table 2). In semisolid cultures prepared with methylcellulose, suppression of colony formation by FD-c-fms cells in cultures stimulated by the combination of CSFs was similar in nature, if not magnitude, to that seen in agar cultures. Thus the use of agar was not mandatory for the suppression, although suppression appeared to be more marked in agar cultures than in other semisolid cultures.

When FD-c-fms were cultured for up to 4 days in suspension in liquid medium, using the same batch of medium and calf serum, the logarithmic accumulation of cells in cultures stimulated by GM-CSF was reduced by the inclusion of M-CSF in the cultures. However, when the data were extrapolated to day 7 of incubation (the standard incubation time used for the semisolid cultures), the reduction in total cell numbers was only 5- to 30-fold, a suppression of lesser magnitude than that usually seen in semisolid agar cultures. Inclusion of a 0.5% agar underlayer beneath the liquid cultures did not increase the magnitude of this reduction.

Table 2. Suppression of FD-c-fms colony formation in various types of semisolid culture

Medium	No. of colonies (cells per colony)		
	GM-CSF	M-CSF	GM-CSF + M-CSF
Agar	93 ± 4 (2530)	73 ± 4 (570)	23 ± 8 (30)
Methyl-cellulose	41 ± 7 (2760)	33 ± 1 (1050)	18 ± 11 (370)
Agar	84 ± 24 (1550)	88 ± 17 (360)	55 ± 2 (80)
Agarose	32 ± 9	3 ± 1	1 ± 0.5

One hundred FD-c-fms cells were cultured in agar, 0.25% agarose, or 1.5% methylcellulose and examined after 7 days of incubation. Mean colony numbers ± standard deviations from four replicate cultures are shown. In parentheses are the mean numbers of cells per colony established from pools of 50 colonies.

Analysis of the Morphology and Membrane Markers of FD-c-fms Cells. Previous studies showed that FDC-P1 or 32D cells expressing M-CSF receptors exhibited monocyte–macrophage differentiation when stimulated by M-CSF (3, 7), a change that was reversible following replacement of M-CSF by multi-CSF. Cells of the present FD-c-fms lines showed a similar size increase compared with parental FDC-P1 cells and a tendency for monocyte–macrophage differentiation when stimulated by M-CSF. In culture for 3–4 days with GM-CSF, the morphology of most cells reverted to that of the parental FDC-P1 cells. In contrast, FD-c-fms cells stimulated by GM-CSF plus M-CSF exhibited a further size increase and more accentuated monocyte–macrophage differentiation with, in various lines, from 5% to 58% of the cells having the morphology of mature macrophages. Flow cytometry revealed that FD-c-fms cells stimulated by M-CSF expressed Mac1 antigen, unlike parental FDC-P1 cells. When stimulated by GM-CSF plus M-CSF, a subset of cells exhibited heightened expression of Mac1. In contrast, the level of expression of GR-1, Mac2, and Mac3 was equivalent on FDC-P1 and FD-c-fms cells regardless of the stimulus used.

Receptor Expression and Internalization by FD-c-fms Cells. Analysis of the binding of ^{125}I-labeled M-CSF to various FD-c-fms cell lines indicated the presence of relatively large numbers of high-affinity M-CSF receptors (4000–6000 per cell). The presence of these additional receptors did not alter the number or affinity of endogenous receptors for GM-CSF. Furthermore, dose–response curves indicated that the six FD-c-fms lines analyzed were equivalent to parental FDC-P1 cells in their responsiveness to stimulation by GM-CSF.

Preincubation of four different FD-c-fms cell lines with either GM-CSF or multi-CSF for 1 hr at 37°C did not reduce the ability of the cells to subsequently bind M-CSF, and preincubation of cells in M-CSF or multi-CSF did not alter subsequent binding of GM-CSF (Table 3). Analysis of the internalization kinetics of ^{125}I-M-CSF at 37°C showed no difference in the kinetics of internalization (k_e = 0.22–0.28 min^{-1}) or intracellular degradation (k_n = 0.01–0.02 min^{-1}) of M-CSF between FD-c-fms cells preincubated for 1 hr with medium, GM-CSF, or multi-CSF (Fig. 4).

Reversion of FD-c-fms Cells. In cultures of some FD-c-fms cell lines stimulated by the combination of GM-CSF and M-CSF, an occasional colony of normal size developed. Such colonies were removed from cultures of two of the cell lines and used to initiate cloned sublines maintained in GM-CSF plus M-CSF. Analysis of these sublines indicated that the

Table 3. Lack of transmodulation of M-CSF or GM-CSF receptors on FD-c-fms clone 5 cells

Preincubation	Specific binding, cpm	
	^{125}I-M-CSF	^{125}I-GM-CSF
Medium	58,000 ± 2,000	6,700 ± 50
GM-CSF	59,000 ± 500	1,800 ± 50
Multi-CSF	61,000 ± 200	7,000 ± 200
M-CSF	11,000 ± 1,200	7,300 ± 250
M-CSF + GM-CSF	13,000 ± 0	1,900 ± 80

FD-c-fms cells (2×10^6 per point) were preincubated for 1 hr at 37°C in medium alone or with the indicated CSF(s) (1 μg/ml), then washed and assessed for ^{125}I-M-CSF or ^{125}I-GM-CSF binding for 3 hr at 4°C. Data are means ± standard errors for duplicate incubations. Three other clones gave similar results.

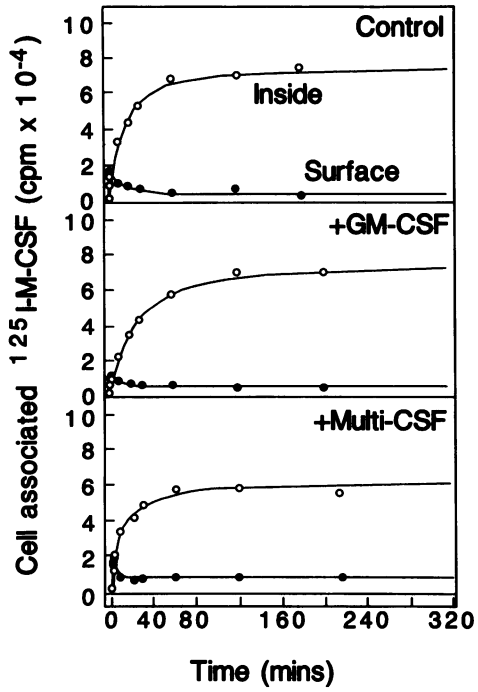

FIG. 4. Internalization kinetics of ^{125}I-M-CSF in FD-c-fms clone 5. Cells (2×10^6 per point) were preincubated for 1 hr at 37°C in medium (control) or in medium containing GM-CSF or multi-CSF (1 μg/ml) before addition of ^{125}I-M-CSF (180,000 cpm, 1.5 nM). At the indicated times, aliquots of cells (duplicate points) were removed for determination of specific cpm on the cell surface (●) or inside the cell (○) by acid dissociation (6). Data were analyzed by a curve-fitting program (6); curves through the experimental points represent lines of best fit.

clonogenic cells were refractory to inhibition by the combined stimulus of GM-CSF plus M-CSF. Although Southern analysis showed the continued presence and integrity of the inserted c-*fms* retroviral construct, Northern analysis showed no expression of the c-*fms* insert (Fig. 5), and incubation of the cells with ^{125}I-M-CSF failed to reveal detectable binding, indicating a failure of the cells to express the M-CSF receptor (c-*fms* product). These sublines therefore appear to be derived from cells in which a shutdown of transcription of the retrovirally inserted c-*fms* had occurred.

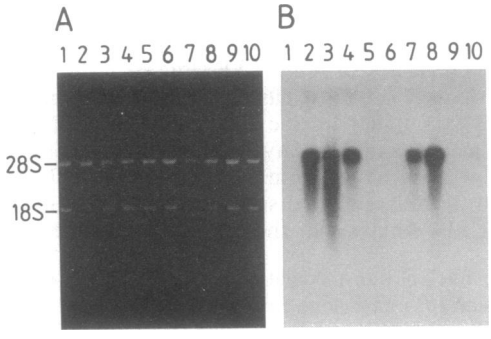

FIG. 5. Northern analysis of FD-c-fms lines and sublines. Poly(A)$^+$ RNA (≈3 μg per lane) was fractionated in a 6% formaldehyde/1% agarose gel, transferred to nitrocellulose, and hybridized with a murine c-*fms* cDNA probe essentially as described (8). Lane 1, FDC-P1 RNA; lane 2, FD-c-fms1; lane 3, FD-c-fms5; lane 4, FD-c-fms6; lanes 5 and 6, sublines of line 6 refractory to inhibition by GM-CSF plus M-CSF; lane 7, FD-c-fms8; lane 8, FD-c-fms9; lanes 9 and 10, sublines of line 9 refractory to inhibition by GM-CSF plus M-CSF. (*A*) Gel stained with ethidium bromide to reveal residual ribosomal RNA; (*B*) Autoradiograph of filter after hybridization. Two slightly different-sized RNAs, corresponding to the spliced and unspliced retroviral transcripts, were evident with shorter exposures.

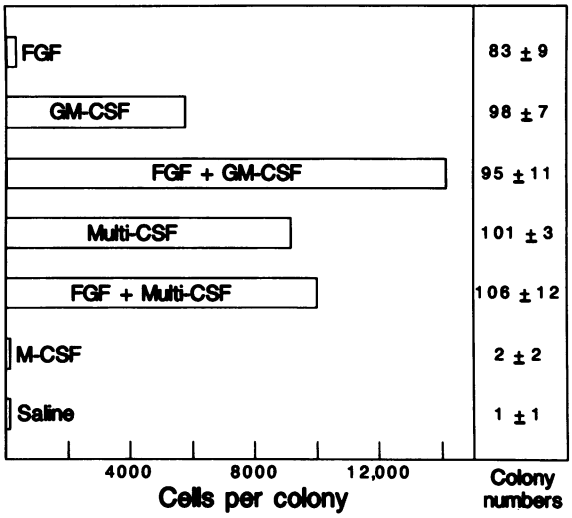

FIG. 6. Colony formation by cells of the FD-FGFR-IL.B line bearing inserted FGF receptors was enhanced when GM-CSF (10^3 units/ml) was combined with FGF (20 ng/ml). Combination of multi-CSF (10^3 units/ml) with FGF was neither inhibitory nor enhancing.

Behavior of FD-FGFR-IL.B Cells. FDC-P1 cells expressing the receptor for basic FGF formed small colonies when stimulated by FGF at 20 ng/ml. Combination of GM-CSF at 1000 units/ml with FGF at 20 ng/ml did not result in a reduction of colony number or size compared with cultures stimulated by GM-CSF alone, and indeed, the combination was found to enhance cell proliferation (Fig. 6). Combination of FGF with multi-CSF did not result in changes in colony number or size compared with cultures stimulated by multi-CSF alone.

DISCUSSION

In cultures of normal mouse marrow cells, combination of GM-CSF with M-CSF leads to an increase in mean colony size (2), and a striking enhancement of colony formation has been observed in corresponding cultures of human cells (9). Despite these overall effects, analysis of murine cultures has indicated that some macrophage colonies in these cultures are suppressed by this combination of growth factors (3).

To develop a simpler model of this phenomenon, we used a cell line normally expressing receptors for GM-CSF and multi-CSF in which expression of M-CSF receptors was induced by retroviral insertion of murine c-*fms* cDNA. With these cells, each CSF alone stimulated proliferation but the cells exhibited marked suppression of proliferative activity when stimulated by even low concentrations of the combination of GM-CSF plus M-CSF and, to a lesser degree, by multi-CSF plus M-CSF. After stimulation by GM-CSF plus M-CSF, the cells exhibited increased monocyte–macrophage differentiation in parallel with an incremental loss of clonogenicity, and it is possible that these two changes are linked.

The phenomenon was not attributable to overstimulation, unusual short-term cross down-modulation of receptors (10), or alteration in the manner in which occupied receptors were internalized (6). Although it has been reported that, in cultures of longer duration, GM-CSF down-modulates the expression of the M-CSF receptors on FD-c-fms cells (3), this phenomenon seems insufficient to account for the present overall suppression, since responsiveness to GM-CSF would be expected to have remained intact. It is unlikely that the suppression is due to a general incompatibility of simultane-

ous signaling from an occupied tyrosine-kinase receptor (M-CSF) (11) combined with that from a non-tyrosine-kinase receptor (GM-CSF or multi-CSF) (12), since combination of FGF (having a tyrosine kinase signaling receptor) with GM-CSF did not result in inhibition. A possibility worthy of exploration is that the suppressive effects of combined CSF signaling may be due to the induction of a special combination of nuclear transcription factors in affected cells that leads to accentuated maturation and an irreversible loss of the capacity to proliferate.

While most FD-c-fms cells were inhibited by the combination of M-CSF plus GM-CSF, occasional cells proliferated well when stimulated by this combination. Analysis showed that these were revertants in which expression of the inserted c-*fms* construct had shut down, a phenomenon we have seen previously in FDC-P1 cells with transcription of a retrovirally inserted construct encoding the human GM-CSF receptor (13). This instability has restricted long-term studies on the possible reversibility of the suppressed phenotype seen with CSF combinations to extend earlier observations on the reversibility of phenotype seen when FD-c-fms or 32D-c-fms cells are grown for a period using only GM-CSF or multi-CSF (3, 4, 7).

Although the FDC-P1 cell line used is immortalized and potentially abnormal in its behavior, it is of some biological interest that the combination of two positive growth factors should be able to suppress cell proliferation. What may be a comparable suppression has also been observed with normal macrophage colonies (2, 3), and the mechanisms responsible need to be clarified, if for no other reason than that the CSFs are in clinical use and there are general grounds for proposing their use in combination (14). Finally, the present suppressive phenomenon is remarkably similar to the unusual ability of the CSFs, alone or in combination, to suppress the clonogenic proliferation of certain myeloid leukemic cell lines (15, 16). Further analysis of the present phenomenon may therefore be of importance for a proper understanding of this potentially valuable action of the CSFs in the clinical management of myeloid leukemia.

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