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Induction of Macrophage Colony-Stimulating Factor-Dependent Growth and Differentiation after Introduction of the Murine c-*fms* Gene into FDC-P1 Cells

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A system has been established for analyzing the functions of the c-*fms*/macrophage colony-stimulating factor (M-CSF) receptor gene product in hematopoietic growth and differentiation. The murine c-*fms* gene was introduced into the factor-dependent murine hematopoietic cell line FDC-P1 by retroviral infection, and conversion to M-CSF-dependent growth was assayed in agar cultures. Expression of the c-*fms* gene in FDC-P1 cells, which normally do not express this gene, resulted in the conversion of resultant FD(c-*fms*) cells to M-CSF-dependent growth. Stimulation of FD(c-*fms*) cells by M-CSF led to the formation of colonies of altered morphology and produced reversible morphological changes suggestive of myeloid differentiation. M-CSF also induced expression of mature myeloid surface marker proteins in the FD(c-*fms*) cells. Neither multi-CSF nor granulocyte-macrophage CSF induced similar phenotypic changes but remained able to stimulate the proliferation of undifferentiated FD(c-*fms*) cells. These results indicate that the c-*fms* gene was expressed functionally in FDC-P1 cells and transmitted signals for growth. Also, the interaction of M-CSF with the c-*fms* gene product generated an additional signal for myeloid differentiation but did not irreversibly commit FD(c-*fms*) cells to terminal differentiation. This system can be used for molecular analysis of the growth- and differentiation-promoting activities of the c-*fms* proto-oncogene.

Hematopoietic cell development is stimulated primarily through the actions of a set of glycoprotein factors that regulate replication, survival, commitment to a specific differentiated lineage and specialized functions of the differentiated cells (25). Multipotential stem cells located in hematopoietic organs (yolk sac, fetal liver, spleen, and bone marrow) self-reproduce and give rise to committed progenitor cells that ultimately, under the influence of the lineage-specific growth factors, develop into one of the multiple families of mature hematopoietic cells. The life span of the mature hematopoietic cells is relatively short, and therefore the stem cells and growth factors provide a continuous flow of differentiating cells along these lineages.

Monocyte-macrophage development is an interesting example of the complexities encountered in the regulation of hematopoiesis. Mature monocytes and macrophages develop from bipotential progenitor cells that can form either granulocytes or monocytes, with the balance being influenced by both the specific growth factor and its concentration (25). No doubt, interplay between and among the different growth factor receptors also may play a role (45). Macrophage colony-stimulating factor (M-CSF; also called CSF-1) is the major lineage-specific growth factor guiding the development along the macrophage arm of the pathway (38, 42) but has some actions on granulocytic cells (25). Granulocyte-macrophage colony-stimulating factor (GM-CSF) can normally stimulate the production of both granulocytes and macrophages but at low concentrations is almost exclusively a macrophage-specific growth factor (5, 25, 45). Granulocyte colony-stimulating factor (G-CSF) functions primarily along the granulocytic lineage but at higher concentrations also leads to macrophage production (6, 29, 45). Thus, macrophages and their precursors contain distinct receptors for M-CSF, GM-CSF, and G-CSF as well as for multi-CSF (interleukin-3), and a hierarchical relationship among these receptors probably exists (45). It is not clear, however, whether each specific receptor imparts a distinct growth and differentiation signal, whether they all utilize the same signaling pathway controlled by receptor expression, or whether one receptor is able to transduce signals by activating alternate receptors.

We would like to understand how the M-CSF receptor functions in the macrophage growth and development scheme. The M-CSF receptor is most evident on mature and immature cells of the monocytic lineage (7, 39) and may play a role in the development and function of trophoblast cells of the placenta (31). The M-CSF receptor is the equivalent of the c-*fms* proto-oncogene protein product (36) and related to the cognate v-*fms* oncogene encoded in the genomes of both Susan McDonough and Hardy-Zuckerman 5 strains of feline sarcoma virus (3, 14). A cDNA to the c-*fms*/M-CSF receptor has been cloned from three species (8, 33, 48), and sequence information has defined several domains of the protein product. The mature form of these cell surface receptor proteins has a carbohydrate-rich extracellular domain of about 490 amino acids (after cleavage of a hydrophobic leader sequence) and contains the M-CSF-binding site amid a region of conserved cysteine residues that help define five immunoglobulinlike domains (48). A transmembrane stretch of 26 hydrophobic amino acids connects the growth factor-binding portion of the protein to the cytoplasmic tyrosine kinase domain and, upon M-CSF binding, presumably conveys a signal that activates this latent tyrosine kinase function (12). A C-terminal segment of up to 50 amino acids probably serves a negative regulatory function for the kinase activity (49), and an insert of approximately 70 hydrophilic amino acids divides the tyrosine kinase homology region. This insert appears to be nonfunctional for kinase activity

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and for fibroblast transformation (41) but may regulate more subtle interactions or a cell-type-specific interaction in macrophages. Upon M-CSF binding and kinase activation, subsequent endocytosis and proteolytic destruction complete the cycle.

The v-*fms* gene product differs from that of the feline c-*fms* in two important respects (49). First, two point mutations in the external receptor-binding domain substitute, at least in part, for genuine M-CSF binding and constitutively transmit an M-CSF-independent signal that turns on the cytoplasmic tyrosine kinase. Second, the 50 C-terminal amino acids encoded in feline c-*fms* are replaced by 11 unrelated amino acids, with the net result that the potential negative regulatory sequence is eliminated. These changes are sufficient to elicit M-CSF-independent growth and cause certain hematopoietic malignancies (16).

We had previously cloned the murine c-*fms* cDNA (33) for the purpose of establishing a system for analyzing the functions and dysfunctions of this growth factor receptor in growth, differentiation, and transformation. To take advantage of the vast knowledge of murine hematopoiesis, and to avoid potential species-specific interactions, we chose the murine model and constructed an ecotropic murine retrovirus for efficient expression of the murine c-*fms* gene (32a). The initial experiments demonstrated that expression of the normal c-*fms* gene in BALB/c fibroblasts resulted in transformation by an autocrine mechanism because M-CSF is produced by these cells.

In this study, we have examined the growth-, commitment-, and differentiation-inducing functions of the c-*fms* gene product by expression of this receptor in a murine hematopoietic precursor cell that represents a more natural environment for this receptor. We selected the FDC-P1 cell line as the target because it is a myeloid precursor and requires either GM-CSF or multi-CSF for growth and does not survive or proliferate in the presence of M-CSF (9, 15). Here we report that introduction of the murine c-*fms* gene into the factor-dependent blast cell line FDC-P1 results in both growth and differentiation in the presence of exogenous M-CSF.

MATERIALS AND METHODS

Cells and culture conditions. FDC-P1 cells were maintained in Dulbecco modified Eagle medium (DME) containing 10% fetal bovine serum and in 10% pokeweed mitogen-stimulated spleen conditioned medium (SCM) as a source of GM-CSF and multi-CSF. ψ2 and virus-producing ψ2 cells were also grown in DME with 10% fetal bovine serum.

Plasmid constructs were introduced into the ψ2 cells by electroporation along with pSV2neo DNA at 1/10 the amount of the retrovirus plasmid (500 V, 25 μF, time constant of 0.4 ms) in the 4-mm electrode cuvettes of a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). The DNAs were added to 1 ml of medium containing 10^6 trypsinized and washed ψ2 cells and placed on ice for 5 min before and 5 min after electroporation. The cells were then diluted into 90 ml of medium, and 3 ml was plated into each 35-mm-diameter well of five six-well plates (Costar, Cambridge, Mass.). G418 selection (400 μg/ml) was started after 2 days. Resistant colonies were grown and tested for c-*fms* protein expression by [^{35}S]methionine labeling and immunoprecipitation. Infectious virus was assayed and titered by focus formation on BALB/c fibroblasts as previously described (32a).

Assays for factor dependence and conversion to M-CSF-dependent growth were performed in 1-ml agar cultures containing final concentrations of 20% fetal bovine serum and 0.3% agar. These assays were performed as described by Metcalf (25) and contained either 1,000 or 300 cells per ml of culture. Human recombinant G-CSF was included in the cultures (240 U/ml) as a negative control for growth, murine GM-CSF (400 U/ml) was the positive control, and murine M-CSF (110 U/ml) served as an indicator of conversion to M-CSF-dependent growth. All growth factors were purified and obtained from Nick Nicola (GM-CSF), DNAX Corp. (G-CSF), or John Hamilton (M-CSF). Assays were performed in quadruplicate, and colony numbers were read after 7 days under a dissecting microscope.

Construction of retroviruses. The pZen(c-*fms*) retrovirus was constructed as previously described (32a) and expressed the murine c-*fms* protein under control of the myeloproliferative sarcoma virus long terminal repeat (4). Virus harvested from ψ2 transfected cells had a titer of 3×10^5 focus-forming units per ml on BALB/c fibroblasts.

A similar Zen-based retrovirus containing the feline v-*fms* oncogene was constructed, starting with the pB5 AluI 1405 insertion mutant of v-*fms* (24). This mutant contained a *Bam*HI linker insertion at nucleotide position 1405 just to the 3′ side of the recombination point between the *gag* and c-*fms* sequences and 5′ of the initiation codon for the c-*fms* gene. This linker mutation permitted the excision of the entire v-*fms* coding sequence as a 3.1-kilobase *Bam*HI fragment and insertion into the *Bam*HI site of the polylinker region of the pJZen-2 retrovirus vector (obtained from J. Chang and G. Johnson, Walter and Eliza Hall Institute). The pJZen-2 vector is identical to the pZen vector into which the murine c-*fms* was inserted but contained a polylinker at the *Xho*I site, and the other *Bam*HI, *Eco*RI, and *Hin*dIII restriction sites in the plasmid were eliminated. Previous results have shown that the *gag* sequences are not necessary for transformation (47), and our results confirmed this observation (data not shown). The v-*fms* retrovirus is termed Zen(v-*fms*), and virus harvested from ψ2 transfected cells exhibited a titer of about 10^5 focus-forming units per ml on BALB/c fibroblasts.

Flow cytometry analysis. Cells were washed in phosphate-buffered saline (PBS) containing 5% fetal bovine serum plus 0.1% sodium azide and stained for 30 min on ice with rat antibodies in the same buffer. Goat immunoglobulin was added to the cells before the immune antibodies to block Fc receptors. The cells were washed in the ice-cold PBS solution between and after antibody applications and analyzed on a FACScan instrument (Becton Dickson Instruments, Palo Alto, Calif.).

Antibodies to the external domain of the murine c-*fms* protein were prepared by injecting Fischer rats with 10^7 Rat-2 cells expressing the murine c-*fms* protein. The animals were boosted twice with 10^7 cells and bled (V. Rothwell and L. R. Rohrschneider, unpublished data). The immune rat serum was diluted 1:20 into PBS–5% fetal bovine serum–0.1% sodium azide, absorbed twice for 1 h each time against 10^7 uninfected FDC-P1 cells, and finally centrifuged at 50,000 rpm in the TL100.2 rotor of a tabletop ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). A 25-μl sample of the diluted and absorbed anti-c-*fms* serum was added directly to 10^6 cells. A secondary fluorescein-conjugated goat anti-rat immunoglobulin [$F(ab')_2$ fragment of AffiniPure and AffiniSorbed antibody] was purchased from Jackson ImmunoResearch Laboratories, Inc., and used at a 1:20 dilution. Rat monoclonal antibody F4/80 was obtained from Peter Klinken at the Walter and Eliza Hall Institute; fluorescein-coupled rat monoclonal antibody to Mac-1, bio-

tinylated rat monoclonal antibody 8C5, and fluorescein-coupled avidin were obtained from Paul Lalor (Walter and Eliza Hall Institute).

Immunoprecipitation and pulse-chase. Cells were labeled for 4 h with [^{35}S]methionine in methionine-free DME containing 5% fetal bovine serum. The cells were placed on ice, washed twice with cold PBS, and extracted with RIPA buffer (24) containing 10 mM EDTA, and labeled proteins in the detergent extract were immunoprecipitated with rabbit anti-murine c-*fms* protein as described previously (50). The rabbit anti-c-*fms* serum was prepared to the region of the tyrosine kinase domain encoded by a 1.17-kilobase *PstI-PstI* restriction fragment. This segment was ligated to a pATH vector; the resultant TrpE–c-*fms* protein was purified from an *Escherichia coli* extract by gel electrophoresis and used to immunize rabbits.

Pulse-chase experiments were performed exactly as described by Lyman and Rohrschneider (24).

RESULTS

FDC-P1 cells were infected with murine ecotropic *fms*-containing retroviruses by cocultivation for 2 days with irradiated virus-producing ψ2 cells in the presence of SCM as a source of growth factors. The retrovirus, Zen(c-*fms*), expressed the murine c-*fms* cDNA from a myeloproliferative sarcoma virus long terminal repeat and was described previously (32a). The Zen(v-*fms*) retrovirus was a similar construct but contained the feline v-*fms* gene. After infection, the FDC-P1 cells were removed from the adherent ψ2 cell layer and washed in PBS to remove surface-bound growth factors. These cells were placed in agar cultures at two densities and grown in the presence of either G-CSF (as a negative control), GM-CSF (as a positive control), or M-CSF to determine successful expression of c-*fms* and conversion to growth on this factor. Two different cell densities were examined to control for possible feeder effects from the stimulated secretion of a growth factor by viral infection. No such effect was observed in any of these experiments. Control infections included cocultivation of FDC-P1 cells with normal ψ2 cells or with ψ2 cells transfected with *neo* alone.

The results of infecting FDC-P1 cells with the *fms* retroviruses are shown in Table 1. Uninfected or mock-infected FDC-P1 cells did not respond to either G-CSF or M-CSF and never exhibited clonal proliferation in soft agar under any circumstances in the presence of these factors. Spontaneous factor-independent colonies also were not observed in the course of more than 20 experiments performed. GM-CSF, however, stimulated 70 to 80% of FDC-P1 cells to generate large compact colonies regardless of whether they were mock infected or infected by either of the Zen retroviruses. In contrast, if the FDC-P1 cells were infected with the Zen(c-*fms*) retrovirus, a small percentage of the cells grew in the presence of M-CSF as the sole growth factor. In general, 1 to 4% of the total number of input FDC-P1 cells were converted to M-CSF-dependent growth. This result suggests that the c-*fms* gene product can be functionally expressed in FDC-P1 cells but with very low efficiency.

The cloning efficiency of c-*fms*-expressing FDC-P1 [i.e., FD(c-*fms*)] cells was examined in soft agar to help determine the cause of the low frequency of conversion to M-CSF-dependent growth. A mass population of c-*fms*-containing FDC-P1 cells was selected immediately after the original infection by growth for 1 week in suspension culture with M-CSF as the only growth factor. These FD(c-*fms*) cells were plated in agar culture with either G-CSF, GM-CSF, or M-CSF as before. No growth occurred in G-CSF, whereas GM-CSF stimulated the same number and size of colonies shown in Table 1. M-CSF also stimulated colony formation, and the cloning efficiency in soft agar was about 10% (data not shown). Because the starting population of FD(c-*fms*) cells was selected in M-CSF and must have expressed the c-*fms* gene product, the low conversion frequency to M-CSF dependence seen in Table 1 was most probably due to the inefficient growth of the M-CSF-stimulated FD(c-*fms*) cells in agar culture. Because 1 to 4% of total Zen(c-*fms*)-infected FDC-P1 cells grew in soft agar immediately after the infection and the cloning efficiency of the mass-selected FD(c-*fms*) cells was 10%, this result indicates that 10 to 40% of the initially infected FDC-P1 cells could have been expressing the c-*fms* protein.

TABLE 1. Conversion of FDC-P1 cells to M-CSF-dependent growth after infection by the Zen(c-*fms*) retrovirus

Virus used for infection	No. of infected or control FDC-P1 cells/ml of culture	Total no. of colonies from cultures grown in the presence of[a]:		
		G-CSF	GM-CSF	M-CSF
Zen(c-*fms*)	1,000	0	768	30
	300	0	270	13
Zen(v-*fms*)	1,000	0	798	0
	300	0	301	0
Control[b]	1,000	0	728	0
	300	0	272	0

[a] Each value represents the average number of colonies from four cultures in one experiment. The total number of experiments varied between three and seven, and each experiment within a group gave similar results.

[b] In the experiment shown, normal ψ2 cells were cocultivated with FDC-P1 cells. Other control cells gave similar results; these included ψ2 cells expressing a deletion mutant of c-*fms* lacking most of the tyrosine kinase domain and G418-resistant ψ2 cell clones.

Infection of FDC-P1 cells with the Zen(v-*fms*) retrovirus did not lead to factor-independent growth after 1 week in agar culture. A similar result has been reported by others (46), but this report indicated that a factor-independent line was obtained after selection in the absence of any growth factor. The v-*fms* oncogene would be expected to stimulate cell growth and transformation and render infected cells M-CSF independent. The mature v-*fms* protein is fully activated for transformation, but M-CSF still binds to the external domain of this receptor and can augment growth stimulation by v-*fms* (23). Even M-CSF, however, did not stimulate growth of FDC-P1 cells infected with Zen(v-*fms*) in our experiments, and selection for factor-independent growth by culturing Zen(v-*fms*)-infected FDC-P1 cells in growth factor-free medium also has not resulted in growth of factor-independent cells (not shown).

The lack of activity of v-*fms* in the FDC-P1 cells could have been due to a low level of expression, to the mutations in v-*fms* that distinguish it from c-*fms*, or to species-specific differences that permit the murine gene to function in the murine FDC-P1 cells but not the feline v-*fms*. The latter two choices are difficult to test; however, we tested the first possibility by comparing the percentage of FDC-P1 cells infected by the Zen(c-*fms*) versus Zen(v-*fms*) retrovirus and the level of *fms* protein expressed on the surface of these cells. FDC-P1 cells were infected with either Zen(c-*fms*) or Zen(v-*fms*) by cocultivation with ψ2 virus-producing cells.

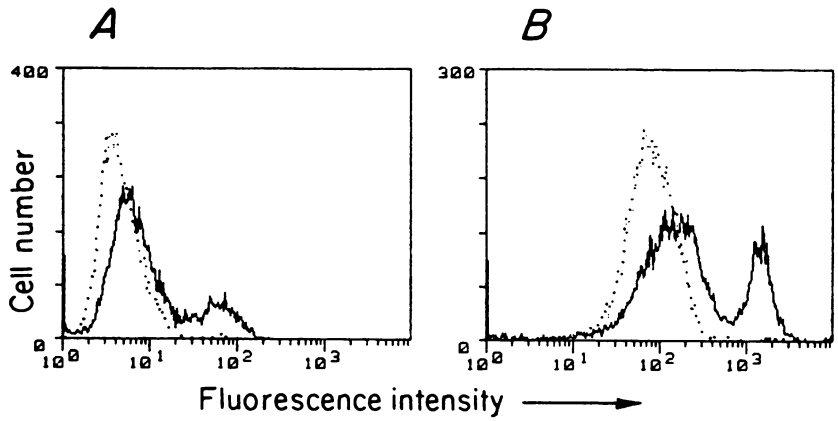

FIG. 1. Determination of the percentage of FDC-P1 cells infected and expressing either the v-*fms* and c-*fms* protein on the cell surface. FDC-P1 cells were infected with either the Zen(v-*fms*) or Zen(c-*fms*) virus by 2-day cocultivation with the irradiated ψ2 virus-producing cells. Each population of cells was then nonselectively expanded in medium containing SCM, and cell surface *fms* determinants were stained with antiserum and a secondary fluorescein-conjugated antibody. The fluorescence intensity was analyzed by flow cytometry. (A) Cells stained with antibody to the v-*fms* protein; (B) cells stained with antibody to the c-*fms* protein. Symbols: . . . . , distribution of stain on uninfected FDC-P1 cells; —— , stain on the Zen(v-*fms*)- and Zen(c-*fms*)-infected cell populations in panels A and B, respectively. The anti-c-*fms* serum produced a high background stain for unknown reasons.

After 2 days, the total population of infected and noninfected FDC-P1 cells was expanded in nonselective medium (i.e., containing SCM) and stained with antibodies to the external domain of the c-*fms* or v-*fms* protein, followed by secondary fluorescein-labeled antibodies. Flow cytometry analysis of these cells (Fig. 1) indicated that in both Zen(v-*fms*)- and Zen(c-*fms*)-infected FDC-P1 cells at least two populations of cells existed. The largest population exhibited less fluorescence but was still more positive than the uninfected FDC-P1 cell controls. This latter group may have been composed of both uninfected cells and infected cells that expressed low levels of the c-*fms* protein. The smaller population was composed of cells with intense fluorescence, indicating high levels of c-*fms* expression. Although it appears that the percentage of FDC-P1 cells expressing higher levels of c-*fms* was much greater than the percentage expressing higher levels of v-*fms*, when the area under each peak was measured, the values were roughly equal. In the FDC-P1 cells infected with the Zen(v-*fms*) virus, the most positive fraction represented 20% of the total, whereas in the Zen(c-*fms*)-infected cells, 25% of the total cell number expressed this higher level of protein. Both v-*fms* and c-*fms* were expressed at higher levels in approximately the same percentage of FDC-P1 cells. Therefore, it does not seem likely that the level of expression could account for the lack of functional v-*fms* in the FDC-P1 cells.

The expression and metabolism of the c-*fms* protein in FD(c-*fms*) cells were examined by labeling FD(c-*fms*) cells with [^{35}S]methionine in the presence of M-CSF and separating immunoprecipitated c-*fms* proteins on a polyacrylamide gel. After a 4-h labeling period, c-*fms* proteins could not be detected in the uninfected FDC-P1 cells, but a broad band of about 140 kilodaltons was found in the FD(c-*fms*) cells, using the anti-c-*fms* serum (Fig. 2, lanes A to E). This protein was probably the product of the murine c-*fms* gene that was either abnormally processed or partially degraded. The latter possibility seemed most likely because higher-molecular-weight c-*fms* proteins were obtained from the same FD(c-*fms*) cells labeled with [^{35}S]methionine in the presence of multi-CSF (Fig. 2, lane F), a growth factor that stimulates replication of FD cells through an alternate receptor. The immature and mature forms of the protein under these

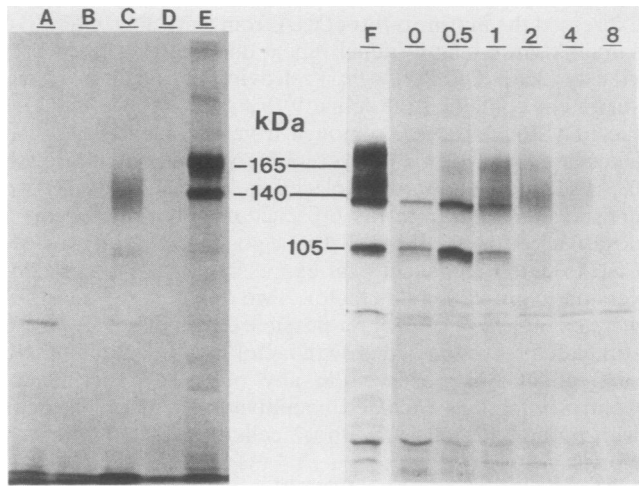

FIG. 2. Immunoprecipitation and pulse-chase analyses to detect expression of the murine c-*fms* proteins in FD(c-*fms*) cells. Cells were labeled for 4 h with [^{35}S]methionine and extracted with detergent; c-*fms* proteins were immunoprecipitated with rabbit antibody prepared to the cytoplasmic domain of the murine c-*fms* protein expressed in *E. coli* and separated by polyacrylamide gel electrophoresis. Shown are the c-*fms* protein products obtained from uninfected FDC-P1 cells with antiserum (lane A) and normal serum (lane B) and from FD(c-*fms*) cells with antiserum (lane C) and normal serum (lane D). Lane E shows the immature (gp140$^{c\text{-}fms}$) and mature (gp165$^{c\text{-}fms}$) products of the murine c-*fms* gene expressed in fibroblasts. The FDC-P1 cells were labeled with [^{35}S]methionine in the presence of 5% serum, and murine M-CSF (100 U/ml) was present during labeling of the FD(c-*fms*) cells. Lane F shows the c-*fms* proteins obtained from FD(c-*fms*) cells labeled with [^{35}S]methionine in the presence of murine multi-CSF (6,800 U/ml). The result of a pulse-chase experiment on the FD(c-*fms*) cells in the presence of murine M-CSF (100 U/ml) is shown in the six rightmost lanes. The number at the top of each lane is the time (in hours) after the initial 25-min pulse of [^{35}S]methionine at which cells were chilled on ice and extracted for immunoprecipitation and gel electrophoresis. The 105-kilodalton (kDa) marker is the approximate size of the nonglycosylated murine c-*fms* primary transcript.

conditions were slightly larger than in the BALB/c-*fms* cells (Fig. 2E), suggesting differences in carbohydrate content.

A pulse-chase experiment also was used to analyze the metabolism of the c-*fms* proteins. The FD(c-*fms*) cells were labeled with [^{35}S]methionine for 25 min in the absence of M-CSF and then chased with an excess of unlabeled methionine plus M-CSF for the times indicated in Fig. 2, and immunoprecipitates were prepared. Both immature (gp140$^{c\text{-}fms}$) and mature (gp165$^{c\text{-}fms}$) forms of the protein were made, and after a 2-h chase the most prominent size of the protein was similar to that seen after long-term label (lane C). This result suggested that the latter protein was a degradation product resulting from rapid endocytosis in the presence of M-CSF. However, we cannot rule out the possibility that long-term growth in M-CSF also affects the carbohydrate content of the mature c-*fms* protein in FD(c-*fms*) cells.

Synthesis of the c-*fms* proteins in the FD(c-*fms*) cells was less efficient than in the BALB/c-*fms* cells (32a). This was evident by the early appearance of the 105- and 135-kilodalton proteins. These were probably the nonglycosylated primary translational product (33) and a glycosylational processing intermediate (27), respectively. This result suggests that the rate-limiting step in processing of c-*fms* proteins within FDC-P1 cells involves an early stage of glycosylational processing. This block may also contribute to the low degree of functional c-*fms* expression in FDC-P1 cells.

The data presented above indicate that expression of the murine c-*fms* gene in FDC-P1 cells can allow the successful transmission of an M-CSF-initiated growth signal with proliferation to form colonies of FD(c-*fms*) cells in soft agar cultures. The morphology of these colonies was of considerable interest. Normally, FDC-P1 cells form compact colonies of undifferentiated cells in the presence of GM-CSF. However, the colonies of Zen(c-*fms*)-infected FDC-P1 cells grown in M-CSF were composed of very dispersed cells. This colony shape was unlike that of the parental FDC-P1 cells and resembled the shape of the dispersed mature myeloid colonies growing in agar cultures of normal bone marrow cells after stimulation by GM-CSF, G-CSF, or M-CSF. The transition from compact colonies to colonies with a corona of dispersed cells or composed wholly of dispersed cells has been documented extensively in studies on the WEHI-3B and M1 leukemic cell lines as a consistent index of the induction of maturation into the granulocytic or macrophage pathways (25).

Several colonies that grew in the M-CSF-containing agar cultures were picked and grown in liquid culture with either GM-CSF or M-CSF as the growth factor to initiate clonal sublines. Fourteen cloned lines were derived, and about half were tested for c-*fms* protein expression by either immunoprecipitation or flow cytometry. All were positive (data not shown). In addition, we could demonstrate that the colony morphology was growth factor dependent. An uncloned population of FD(c-*fms*) cells produced compact colonies in agar cultures containing GM-CSF and dispersed colonies in cultures containing M-CSF (Fig. 3). This result suggests that the interaction of M-CSF with the c-*fms* gene product not only stimulates growth but also alters the cellular phenotype.

The morphology of FD(c-*fms*) cells growing in cultures stimulated by multi-CSF or GM-CSF differed slightly from that of FD cells (Table 2) in that a small percentage of cells had ring-shaped nuclei or the morphology of immature or mature macrophages. Some of the cells had small numbers of cytoplasmic granules resembling the primary granules of promyelocytes. FD(c-*fms*) cells growing in cultures stimulated by M-CSF differed dramatically in morphology. A larger percentage of cells had a macrophagelike morphology, and more than 90% of cells, including those with macrophage morphology, exhibited large numbers of primary granules. This major morphological difference was reversible if cells grown in M-CSF were washed and then grown in multi-CSF. Similarly, cells grown initially in multi-CSF acquired the high content of granules when grown in M-CSF. The morphology of these cells is shown in Fig. 4, and quantitative information given in Table 2.

Other potential changes in the cellular phenotype of the M-CSF-stimulated FD(c-*fms*) cells were examined by flow cytometry. We looked at the cell surface expression of the c-*fms* protein as well as other specific marker proteins of differentiated myeloid cells. The c-*fms* protein was expressed on the surfaces of both a mass population and a specific clone of FD(c-*fms*) cells, respectively (Fig. 5A and B). The uninfected FDC-P1 cells did not express the c-*fms* protein, but nonspecific staining with the anti-c-*fms* serum was relatively high and not absorbed out by uninfected FDC-P1 cells. This background fluorescence did not, however, obscure the specific staining. The mass culture of FD(c-*fms*) cells contained two populations exhibiting differing levels of membrane c-*fms* protein, whereas the FD(c-*fms*) clone was uniformly high in expression. Other FD(c-*fms*) clones have also been uniformly high in c-*fms* protein expression, but some have contained the two populations seen in Fig. 5A (data not shown). We do not yet know whether this result reflects the bifurcated differentiation pathway (granulocyte versus macrophage) or whether it is a characteristic of each particular clone, the growth factor concentration, or perhaps some other uncontrolled factor.

The expression of additional myeloid cell surface markers on the FD(c-*fms*) cells was examined by flow cytometry. Monoclonal antibodies were used to detect the Mac-1 antigen, and two additional lineage-restricted antigens were assayed by monoclonal antibodies RB6-8C5 and F4/80. The Mac-1 antigen (also called Ly-40 or CD11b) is expressed by both monocytes-macrophages and granulocytes, is a member of the integrin family of cell surface adhesion molecules (20), and functions as the type three complement receptor (34). The antibody designated RB6-8C5 is specific for mature granulocytes (17), whereas the F4/80 antibody reacts with monocyte-macrophage-specific determinants (1, 19, 22). The levels of expression of the three epitopes described above on uninfected FDC-P1 cells and on FD(c-*fms*) cells grown in M-CSF are shown in Fig. 5C to H. Mac-1 was not present on uninfected FDC-P1 cells, but its expression increased dramatically in the FD(c-*fms*) cells grown in M-CSF (Fig. 5C and D). Similarly, the antigen marker detected by RB6-8C5 also increased after infection but not to the same degree as Mac-1 (Fig. 5E and F). Five additional clones of FD(c-*fms*) cells were analyzed, and all were positive, to various degrees, for Mac-1 and 8C5. F4/80 had previously been shown to be expressed on normal FDC-P1 cells (15). Our analysis confirmed this observation and demonstrated that there was no change in F4/80 expression on the M-CSF-grown FD(c-*fms*) cells (Fig. 5G and H).

Expression of the new surface markers on the FD(c-*fms*) cells required the continuous presence of M-CSF. This was demonstrated by analysis of surface markers after the reciprocal shift of FD(c-*fms*) between medium containing either multi-CSF or M-CSF. A clone of FD(c-*fms*) cells was maintained in medium containing GM-CSF and then washed and shifted to medium containing either multi-CSF or M-

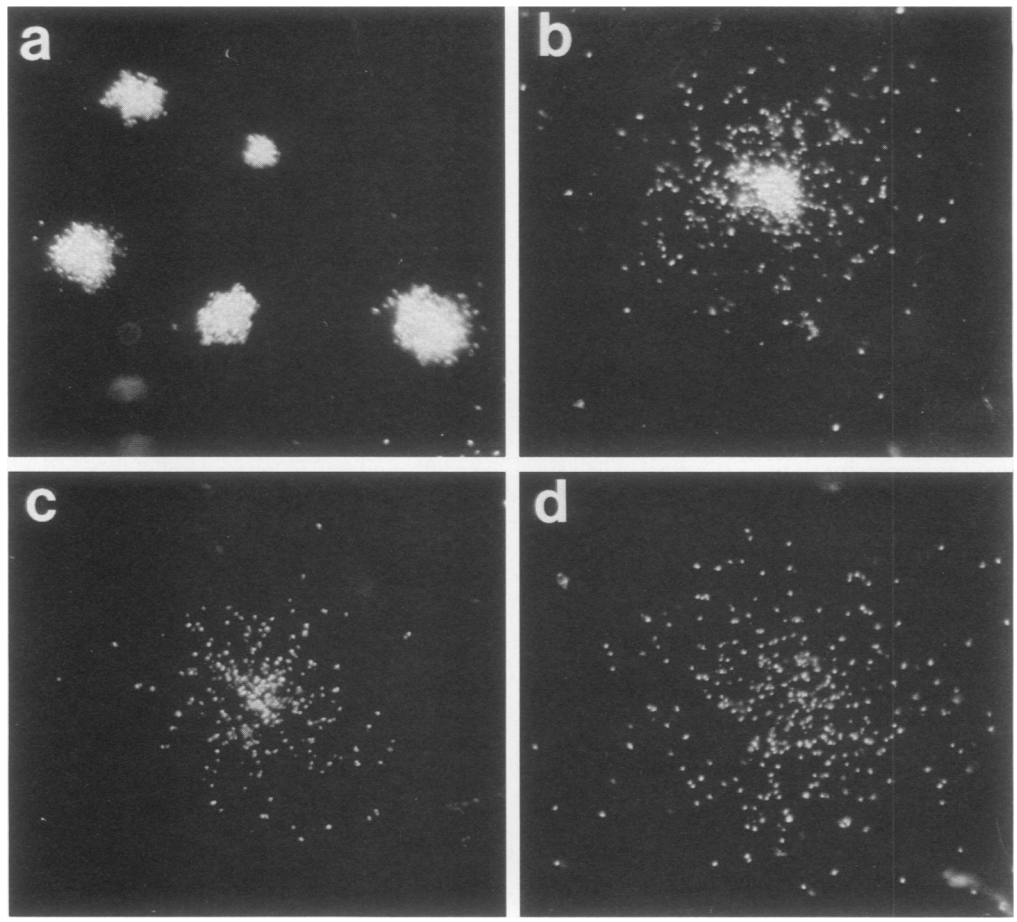

FIG. 3. Colony morphology of FD(c-*fms*) cells grown in soft agar containing either GM-CSF or M-CSF. A population of FD(c-*fms*) cells was selected by growth (6 days) in M-CSF after infection of FDC-P1 cells by the Zen(c-*fms*) virus during the 2-day cocultivation with the ψ2 virus-producing cells. These FD(c-*fms*) cells were washed free of growth factor and grown in soft agar cultures with either 1,500 U of GM-CSF per ml (a) or 200 U of M-CSF per ml (b to d).

CSF (Fig. 6A). After a 1-week growth phase in either M-CSF or multi-CSF, the cells were tested for the expression of c-*fms* protein, Mac-1, and 8C5 antigens by flow cytometry (Fig. 6B). The remainder of the cells grown in multi-CSF were washed free of growth factor and placed in medium containing M-CSF. Likewise, the M-CSF-grown cells were shifted to multi-CSF-containing medium. After a second 5-day phase of growth, expression of the same surface markers was analyzed by flow cytometry (Fig. 6B). The results demonstrate that the expression of Mac-1 and 8C5 antigens on the FD(c-*fms*) cells was reversible and dependent on the continuous presence of M-CSF. Cells grown in multi-CSF did not maintain the expression of these differentiation-specific antigens even after initial growth in M-CSF. The reversible nature of this phenomenon also indicated that M-CSF induced these antigens within the total cell population rather than selecting and expanding a small proportion of antigen-positive cells.

TABLE 2. Morphological changes in FD(c-*fms*) cells after stimulation by M-CSF

Cell type	Stimulus	% of cells[a]			With cytoplasmic granules[b]
		Blasts	With ring nuclei	Macrophages	
FD	Multi-CSF	98	2	0	0
FD(c-*fms*)	Multi-CSF	90	6	4	16
	GM-CSF	92	4	4	15
	M-CSF	82	1	17	94
	M-CSF→ multi-CSF	96	1	3	5
	Multi-CSF→ M-CSF	85	1	14	89

[a] Cells with ring nuclei had the general characteristics of blast cells. Cells classified as macrophages ranged in morphology from promonocytes to mature macrophages with bulky vacuolated cytoplasm.

[b] Determined from examination of sequential cells regardless of morphology.

Cell growth curves also indicated that the FD(c-*fms*) cells could be switched between growth factors without substantial loss of viability (Fig. 7). FD(c-*fms*) grew best with GM-CSF in the medium and initiated growth without delay, but with a slightly slower overall growth rate, when shifted to medium containing only multi-CSF as the growth factor. FD(c-*fms*) cells shifted from GM-CSF to M-CSF showed a slight lag in growth but resumed growth at the same rate as cells grown in multi-CSF. The reciprocal shift of FD(c-*fms*) cells from multi-CSF to M-CSF and from M-CSF to multi-CSF resulted in a longer lag phase; however, growth resumed at the same rate as before the shift. Inspection of the cultures under the microscope indicated that cell death was not the major cause of the lag in growth.

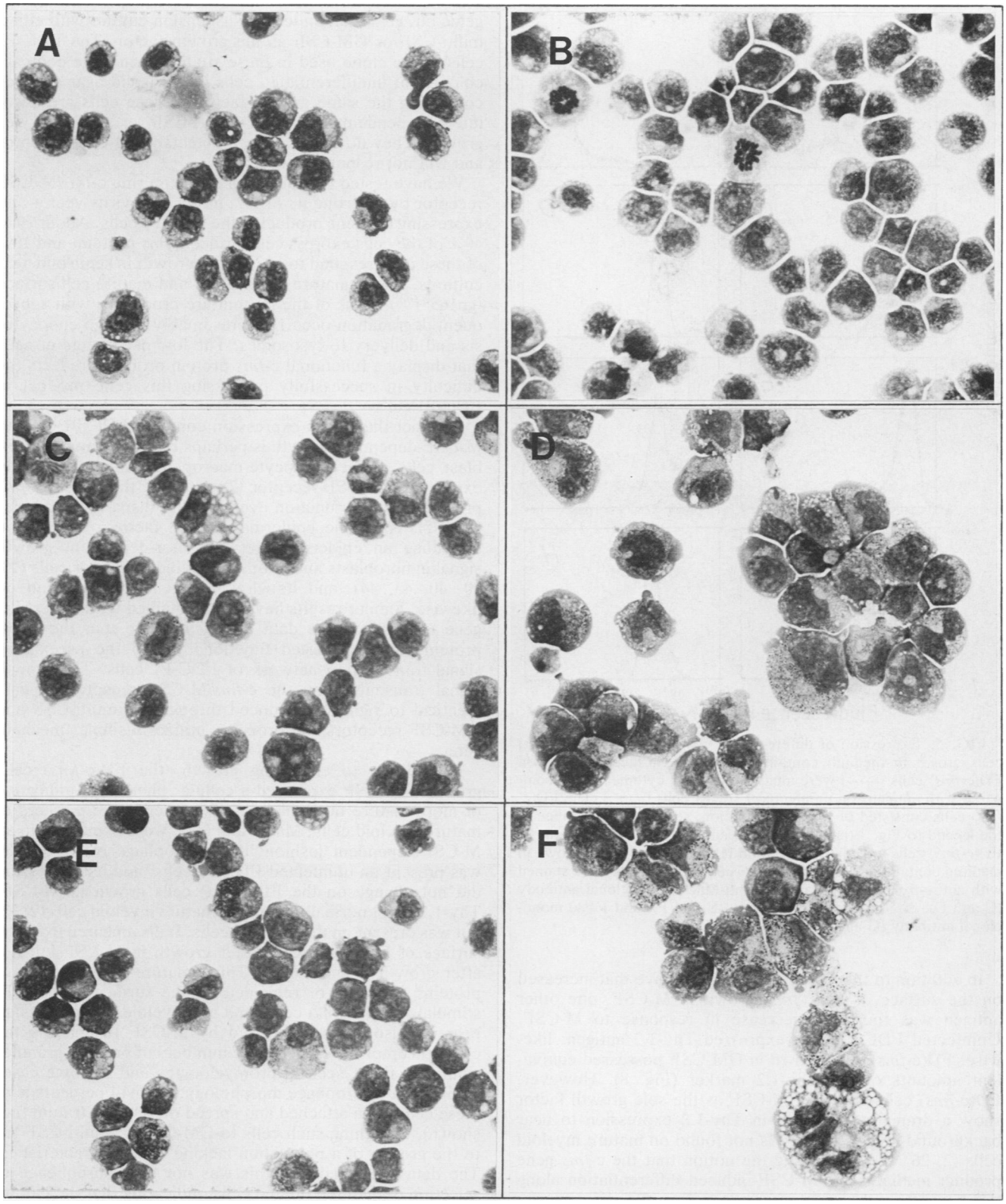

FIG. 4. Morphology of FDC-P1 cells and FD(c-*fms*) cells stimulated by various growth factors. Cells were grown for at least 5 days in the indicated growth factor before cytospins were stained with May-Grunwald-Giemsa stain and hematoxylin. (A) Uninfected FDC-P1 cells maintained in a culture containing multi-CSF (100 U/ml); (B) FD(c-*fms*) cells from a culture stimulated by GM-CSF (400 U/ml); (C) FD(c-*fms*) cells from a culture stimulated by multi-CSF (100 U/ml); (D) FD(c-*fms*) cells from a culture stimulated by M-CSF (2,000 U/ml); (E) FD(c-*fms*) cells grown in M-CSF (2,000 U/ml) and then washed and shifted to growth medium containing multi-CSF (100 U/ml); (F) FD(c-*fms*) cells grown in multi-CSF (100 U/ml) and then washed and shifted to growth medium containing M-CSF (2,000 U/ml). The protocol for growth of the cells was identical to that described in the legend to Fig. 6. Granules can be seen in the cytoplasm of many cells shown in panels D and F, and enlarged vacuolated macrophage-type cells are evident.

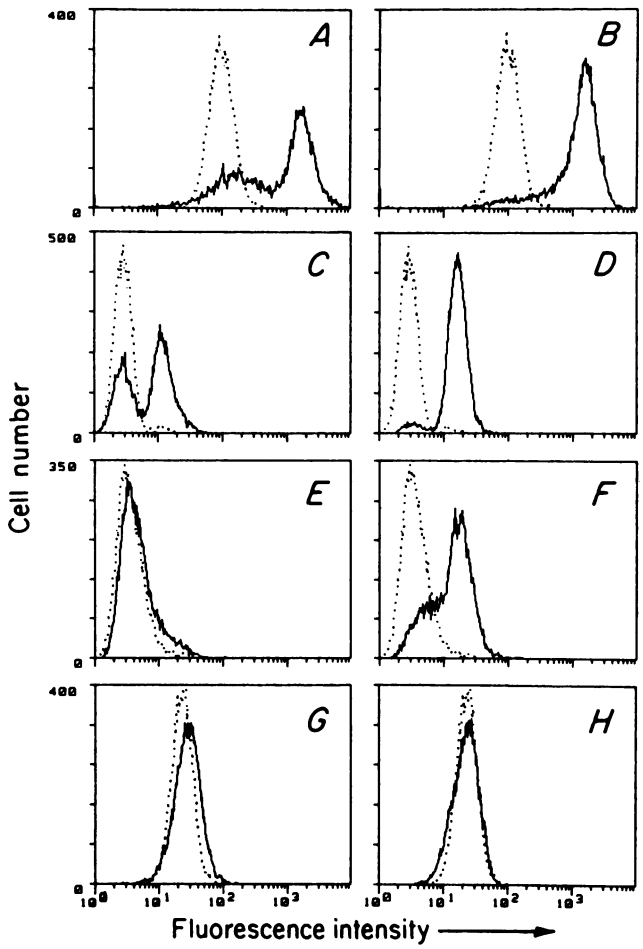

FIG. 5. Expression of differentiated cell markers on FD(c-*fms*) cells grown in medium containing M-CSF. Surface antigens on FD(c-*fms*) cells (——) were analyzed by flow cytometry and compared with antigens on uninfected FDC-P1 cells (. . . .). The FD(c-*fms*) cells consisted of a mass population, selected as described in the legend to Fig. 3 (panels A, C, E, and G), and a single clone of FD(c-*fms*) cells (panels B, D, F, and H). These cells were grown in medium containing 200 U of M-CSF per ml. The cells were stained with anti-c-*fms* serum (A and B), anti-Mac-1 monoclonal antibody (C and D), 8C5 monoclonal antibody (E and F), and F4/80 monoclonal antibody (G and H).

In addition to the markers described above that increased on the surface of FD(c-*fms*) grown in M-CSF, one other antigen was found to decrease in response to M-CSF. Uninfected FDC-P1 cells expressed Thy-1.2 antigen; likewise, FD(c-*fms*) cells grown in GM-CSF possessed equivalent amounts of the Thy-1.2 marker (Fig. 8). However, FD(c-*fms*) cells grown in M-CSF as the sole growth factor show a dramatic reduction in Thy-1.2 expression to near background levels. Thy-1.2 is not found on mature myeloid cells (2, 26, 32), supporting the notion that the c-*fms* gene product mediates the M-CSF-induced differentiation along this lineage.

DISCUSSION

FDC-P1 cells were derived from a long-term bone marrow culture maintained in multi-CSF and represent a population of committed myeloid progenitor cells with the capacity for extensive self-renewal (9, 15). These cells are nontumorigenic but grow indefinitely in suspension culture with either multi-CSF or GM-CSF as the growth factor. The FDC-P1 cells of the clone used in these studies form large compact colonies of undifferentiated cells in semisolid agar cultures containing the same growth factors. These cells are absolutely dependent on either multi-CSF or GM-CSF for growth. They do not express a detectable M-CSF receptor and will not respond to M-CSF.

We have tested the functions of the murine c-*fms*/M-CSF receptor by inserting its cDNA into a retrovirus vector and expressing the gene product in the FDC-P1 cells. About 20 to 40% of the cells express cell surface c-*fms* protein, and 10% of these cells respond to M-CSF by growth in semisolid agar cultures. The immature ($gp140^{c\text{-}fms}$) and mature cell surface ($gp165^{c\text{-}fms}$) forms of the protein are produced, with subsequent degradation occurring presumably through endocytosis and delivery to lysosomes. The low percentage of cells that display a functional c-*fms* protein probably reflects the difficulty in successfully processing this gene product in FDC-P1 cells.

The fact that c-*fms* expression converts FDC-P1 cells to M-CSF-dependent growth is perhaps not surprising because blast cells in the monocyte-macrophage lineage normally express the M-CSF receptor (7); after all, the related v-*fms* protein seems to function even in more disparate cell types (16, 35). Also, the epidermal growth factor receptor can transduce an epidermal growth factor-dependent growth signal in fibroblasts and in other myeloid precursor cells (10, 30, 40, 43, 44), and its viral oncogene, v-*erbB*, can do likewise. Similar results have been obtained with the *erbB-2* gene (11, 18). These data clearly indicate that the c-*fms* protein can be inserted functionally into the preexisting signal transduction network of FDC-P1 cells. The growth signal transmitted by the c-*fms*/M-CSF receptor appears identical to signals transduced through the multi-CSF and GM-CSF receptors and probably utilizes a similar mechanism.

In addition to exhibiting growth, the FD(c-*fms*) cells grown in M-CSF expressed a cellular phenotype indicative of more mature myeloid cells. Two membrane markers of mature myeloid cells, Mac-1 and 8C5, were expressed in an M-CSF-dependent fashion; the macrophage marker F4/80 was present on uninfected FDC-P1 cells, and its expression did not change on the FD(c-*fms*) cells grown in M-CSF. Thy-1.2 is not normally found on mature myeloid cells (2, 32) but was present on the FDC-P1 cells. It disappeared from the surface of FD(c-*fms*) cells after growth in M-CSF but not after growth in multi-CSF. Thus, mature myeloid marker proteins appeared or remained on the surface of M-CSF-stimulated FD(c-*fms*) cells, and nonmyeloid markers disappeared. Also, cells stimulated by M-CSF to activate the c-*fms* receptor developed large numbers of primary granules similar to those seen in promyelocytes and in some cases exhibited a macrophage morphology. A small percentage of these cells even attached and spread on the substratum (not shown). Switching such cells to GM-CSF or multi-CSF led to the growth of a population lacking these characteristics. The data suggested that this was not the consequence of selection of undifferentiated cells, indicating that the parameters of differentiation discussed above were reversible on deactivation of the c-*fms* receptor.

The differentiation was specifically initiated and maintained by the introduced c-*fms* gene in the presence of the M-CSF ligand. Insertion of c-*fms* in itself did induce some change in cellular morphology, but this was relatively minor so long as the M-CSF receptors were not used as the signal

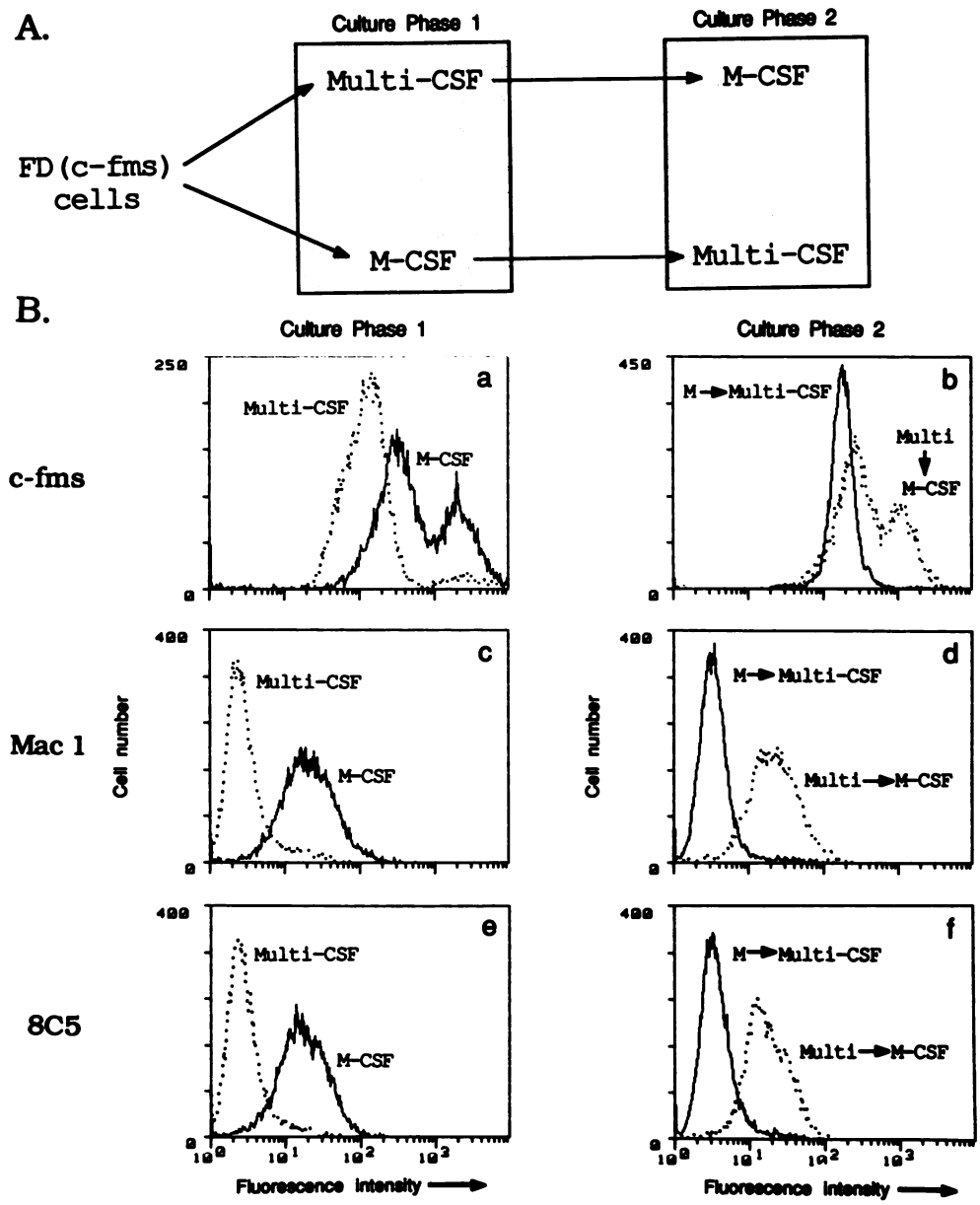

FIG. 6. Demonstration that expression of differentiated cell markers on FD(c-*fms*) is growth factor dependent. (A) FD(c-*fms*) clone 2 cells were grown and maintained in medium containing 400 U of GM-CSF per ml and then washed free of growth factor, placed in medium containing either multi-CSF (100 U/ml) or M-CSF (2,000 U/ml), and grown for 7 days (phase 1 culture). The cells grown in multi-CSF were washed free of growth factor and placed in medium containing M-CSF; conversely, the M-CSF-grown cells were washed and placed in multi-CSF-containing medium. Growth of the cells in this second culture phase was for 5 days. (B) At the end of each culture phase, FD(c-*fms*) cells were analyzed by flow cytometry for expression of c-*fms* (graphs a and b) and for cell surface markers detected by anti-Mac-1 monoclonal antibody (graphs c and d) and 8C5 monoclonal antibody (graphs e and f). Symbols: ——, cells grown in M-CSF during phase 1 and shifted to multi-CSF in phase 2; . . . . , cells grown in multi-CSF for phase 1 and shifted to M-CSF in phase 2.

mechanism to induce proliferation. The FD(c-*fms*) cells also expressed receptors for multi-CSF, GM-CSF, and M-CSF and replicated in the presence of either of these factors. Both multi-CSF and GM-CSF can stimulate myelopoiesis and produce differentiated colonies of granulocytes and macrophages from bone marrow cells; however, in the FD(c-*fms*) cells, only M-CSF evoked a differentiation response. This finding suggests that, at least in the FD(c-*fms*) cells, the M-CSF-induced pathway for growth stimulation is separate from that leading to differentiation. Alternatively, the c-*fms* protein may transmit inseparable growth and differentiation signals distinct from the growth signal initiated from the multi- and GM-CSF receptors.

We also have observed that multi-CSF induces *trans* down-modulation of the c-*fms* protein in FD(c-*fms*) cells (B. Gliniak and L. R. Rohrschneider, unpublished observation). This was evident in the flow cytometry analysis of FD(c-*fms*) cells maintained in multi-CSF for more than 24 h (Fig. 6B, graphs a and b) but not after short-term exposure to multi-CSF (Fig. 2, lanes C and F). Such a mechanism has been proposed to explain the broad spectrum of CSF activities (28, 45), but in our experiments the *trans* down-modulation

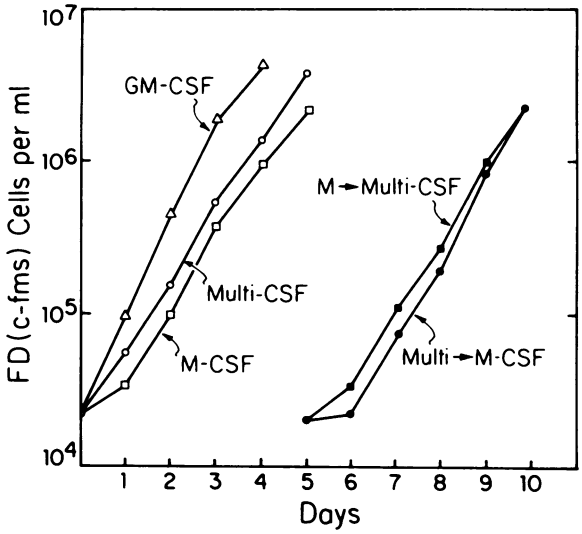

FIG. 7. Growth curves of FD(c-*fms*) cells measuring adaptation to the various growth factors. FD(c-*fms*) clone 5.1 cells maintained in suspension culture with GM-CSF were washed free of growth factor, and samples were initiated on day 0 in GM-CSF (200 U/ml), multi-CSF (100 U/ml), or M-CSF (1,000 U/ml). Cell counts were made daily thereafter. On day 5, the cells in M-CSF and multi-CSF were washed free of growth factor and diluted, and culture was reinitiated in the reciprocal growth factor at the concentrations given above. Cells were again counted daily. All growth factors were derived from the mouse.

of the c-*fms* protein was much slower than the modulation induced by M-CSF and did not induce the same differentiation function. Therefore, the model that other growth factors may regulate differentiation by transmodulation of the M-CSF receptor does not fit the results obtained with the FD(c-*fms*) cells; however, we cannot rule out the possibility that the FDC-P1 cells are defective in this coupling mechanism.

A dominant property of hematopoietic growth factors is their ability to commit progenitor cells to a restricted cell

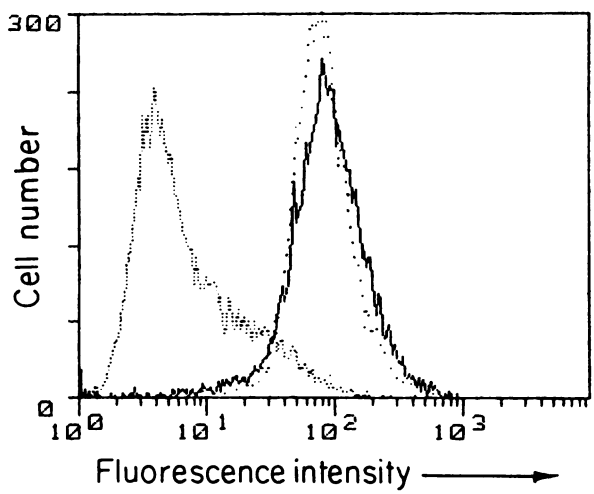

FIG. 8. Expression of Thy-1.2 on FDC-P1 and FD(c-*fms*) cells detected by flow cytometry. A monoclonal antibody to Thy-1.2 antigen was used to stain uninfected FDC-P1 cells (. . . .), FD(c-*fms*) cells grown in medium containing 100 U of multi-CSF per ml (——), or FD(c-*fms*) cells grown in medium containing 640 U of M-CSF per ml (·····).

lineage. Two features of this commitment step in the FD(c-*fms*) cells were of interest. First, it required the continuous presence of M-CSF and could be totally reversed by removal of the M-CSF and growth of the cells in multi-CSF. This finding suggests that additional steps may be required to irreversibly commit cells to a single lineage. The second feature of interest was that, for the most part, the FD(c-*fms*) cells became committed to the monocyte-macrophage lineage in the presence of M-CSF but did express one granulocytic antigen detected by monoclonal antibody RB6-8C5 (17) and granules more typical of promyelocytes. The expression of the granulocytic markers on a predominantly monocyte background may reflect the abnormal expression of some markers reported in hematopoietic differentiation and leukemia (13, 37). Alternatively, M-CSF does have some ability to stimulate granulocytic cells (25), and therefore induction by c-*fms* of some parameters of granulocytic differentiation is not of necessity abnormal. However, the response was unusual in its magnitude and abnormal where granule development occurred in cells with the morphology of mature macrophages. Here a more likely explanation is that the FDC-P1 cells are abnormally programmed rather than that the inappropriate expression and activation of the c-*fms* receptor was the direct cause of the anomaly. The karyotype and immortality of FDC-P1 cells clearly suggest potential abnormalities (21).

Hematopoietic cell malignancies consist of populations of precursor cells blocked in their normal differentiation pathway but with sustained proliferative capacity. It is therefore logical to propose that mutations in c-*fms* that cause oncogenic activation might destroy the ability of c-*fms* to stimulate differentiation but still promote proliferation of undifferentiated cells. An alternative possibility is that mutations in c-*fms* destroy both growth and differentiation activities, with immature precursor cell proliferation driven by an alternate growth factor. It is possible to test these mechanisms of hematopoietic cell transformation in the FDC-P1 cell assay by determining whether growth- and differentiation-promoting activities of the murine c-*fms* gene can be dissociated by introducing mutations that we have identified as causing oncogenic activation (49). These future experiments will permit dissecting the function of individual and combinations of mutations and serve as a potential paradigm for human hematopoietic cell malignancies.

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