Premature Expression of the Macrophage Colony-stimulating Factor Receptor on a Multipotential Stem Cell Line Does Not Alter Differentiation Lineages Controlled by Stromal Cells Used for Coculture

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Summary

We are interested to know whether expression of a lineage-specific growth factor receptor is deterministic to lineage commitment during hematopoiesis. For this purpose, we introduced the human *c-fms* gene into the multipotential stem cell clone LyD9 and two myeloid progenitor clones, L-GM3 and L-G3, cells that differentiate in response to granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte (G)-CSF, respectively. Although LyD9 cells have differentiation potential to become macrophages, *c-fms* transfectants of LyD9 and L-GM3 cells did not differentiate in response to human macrophage (M)-CSF. However, *c-fms* transfectants of L-G3 cells differentiated to neutrophils in response to human M-CSF. These results indicate that the M-CSF receptor requires a specific signal transduction pathway to exert its differentiational and proliferative effects. Furthermore, the M-CSF receptor can convey a granulocyte-type differentiation signal possibly by cooperating with the G-CSF receptor signal transduction pathway. The *c-fms*-transfected LyD9 cells as well as the original LyD9 cells differentiated predominantly into GM-CSF- and G-CSF-responsive cells by coculturing with PA6 and ST2 stromal cells, respectively. The results indicate that differentiation lineage is not affected by premature expression of the M-CSF receptor. Instead, the stromal cell used for coculture apparently controls lineage-selective differentiation of the multi-potential stem cell line.

A line of evidence has shown that hematopoiesis is regulated by a set of growth factors that bind to specific receptors expressed on the surface of progenitors as well as mature blood cells (1). Especially granulocyte/macrophage colony-stimulating factor (GM-CSF),[1] granulocyte CSF (G-CSF), and macrophage CSF (M-CSF) can induce proliferation and differentiation of each lineage cell. For example, the number of neutrophils is strongly augmented by administration of G-CSF (2). Mutant mice that have a defect in the M-CSF gene have few macrophages (3). Progenitor cells that can respond to these lineage-specific CSF appear committed already to each lineage. Progenitor cells express specific CSF receptors that can transduce growth and differentiation signal. Commitment of a multipotential stem cell to myeloid progenitor cells is likely to involve the biochemical events that induce expression of the lineage-specific CSF receptor.

Hematopoiesis is also regulated by direct contact of stem cells with stromal cells. In a long-term bone marrow culture system, hematopoiesis of various lineage cells can be sustained by bone marrow stromal cells without exogenous growth factors (4–6). We have recently shown that coculture of a multipotential stem cell line, LyD9, with the PA6 and ST2 stromal cell lines (7, 8) can support differentiation into GM-CSF-responding and G-CSF-responding cells, respectively (9). The GM-CSF-responsive clone (L-GM3) thus obtained differentiated into macrophages and neutrophils in response to GM-CSF. The G-CSF-responsive cell clone (L-G3) differentiated into neutrophils in response to G-CSF. Neither the PA6 nor the ST2 line was able to support differentiation of M-CSF-responsive cells, although M-CSF was produced by both PA6 and ST2 lines (10). These results indicate that products secreted from or expressed on the stromal cells might play decisive roles in induction of CSF-specific functional receptors on multipotential cells.

Conveyance of regulatory signals by growth factors depends on establishment of at least three biochemical systems: (*a*)

[1] *Abbreviation used in this paper:* GM-CSF, granulocyte/macrophage colony-stimulating factor.

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expression of specific receptors, (*b*) cytoplasmic signal transduction pathways, and (*c*) chromatin opening or transcription competency of the growth factor–responsive genes. We wish to know whether the expression of receptors for lineage-specific growth factors affect differentiation and commitment. For this purpose, we have chosen the human M-CSF receptor as the receptor by the following reasons: (*a*) M-CSF almost exclusively increases macrophage lineage cells; (*b*) the single chain of the M-CSF receptor encoded by the human c-*fms* gene (11) is fully active for signal transduction (12); and (*c*) as human M-CSF receptor does not bind murine M-CSF, we can avoid autocrine growth as well as selective expansion of M-CSF-responding cells until we add human M-CSF. Throughout this study, human M-CSF was used unless otherwise indicated.

We have introduced the human M-CSF receptor gene into LyD9, L-GM3, and L-G3 cells, and tested whether M-CSF could direct differentiation of c-*fms* transfectants of LyD9 cells and its derivatives into macrophages. We found that the M-CSF receptor expression alone did not allow these cells to differentiate into macrophages in response to M-CSF. We also found that premature expression of the M-CSF receptor on LyD9 and L-GM3 cells could not affect the differentiation lineage that is regulated by the specific stroma cell lines (PA6 and ST2) used for the coculture. These results indicate that expression of a lineage-specific growth factor receptor alone is not sufficient for lineage commitment of the multipotential cell and that the stromal cell used for coculture apparently controls differentiation lineages.

Materials and Methods

Cytokines. rIL-3, rIL-4, and rIL-5 were obtained from culture supernatants of X63Ag8 myeloma cells transfected with murine IL-3, IL-4, and IL-5 cDNA, respectively (13). One unit of IL-3, IL-4, and IL-5 was defined as the amounts of the supernatants that gave a half of the maximal proliferative response to LyD9, K4 (14), and K5 cells (15), respectively. Murine rGM-CSF and rG-CSF were culture supernatants of CHO cells transfected with respective cDNAs, which were kindly provided by Dr. T. Sudo (Biomaterial Institute Co. Ltd., Kanagawa, Japan). The activities of GM-CSF and G-CSF were measured using IC-2 and NFS-60 cells. Human rM-CSF was purchased from Genzyme (Boston, MA).

Cell Lines. The procedures for the establishment and detailed characterization of LyD9, LS-1, L-GM, and L-G were described (9, 14, 16). LS-1, L-GM3, and L-G3 were established as clones by limiting dilution of LS, L-GM, and L-G cell lines. LS-1, L-GM3, and L-G3 cells have indistinguishable phenotypes of parent cell lines L-GM and L-G, respectively. All of them were maintained in the complete medium (RPMI 1640, 10% FCS, 50 μM 2-ME, 2 mM L-glutamine) supplemented with rIL-3 (10 U/ml). Stromal cell clones ST2-S10 (ST2) and MC3T3-G2/PA6 (PA6) were established from bone marrow of NZB mice and maintained in the RPMI 1640 containing 5% FCS and 50 μM 2-ME.

Coculture with Stroma Cells. The detailed methods and results of the coculture of LyD9 and stromal cells PA6 and ST2 were described (9). Before coculture with stroma cells, c-*fms*-expressing cells to be cocultured with PA6 cells were treated with 5-azacytidine in the following way. 5-azacytidine was added to 5 μg/ml in 5 ml IL-3-containing complete medium with 10^6 cells/ml. After 24 h, 5 ml of fresh medium without 5-azacytidine was added and maintained for 3 d. Cells were washed and expanded with rIL-3 for the coculture. It was demonstrated that GM-CSF-responding cells were generated with coculture of PA6 without 5-azacytidine treatment, but this treatment promoted the generation of CSF-responding cells. Coculture was performed in T25 flasks (Corning Glass Works, Corning, NY) containing monolayers of PA6 or ST2 cells. c-*fms*-expressing cells were added (5×10^5 cells/flasks) in 5 ml of the complete medium supplemented with IL-3 (1–3 U/ml). The medium was half changed every 4 d with the same medium. After 2 wk of the coculture, cells cocultured with PA6 and ST2 stromal cells were harvested and transferred to rGM-CSF (20 U/ml) or rG-CSF (20 U/ml) containing medium, respectively. Cells were cultured further for 3 d, and then expanded with IL-3. GM-CSF-responding lines LyD9/*fms*-1/GM and LyD9/*fms*-2/GM were obtained from LyD9/*fms*-1 and LyD9/*fms*-2, respectively. G-CSF-responsive cell line LyD9/*fms*-1/G was obtained from LyD9/*fms*-1 cells. LyD9/*fms*-2/GM were further cocultured with ST2 as described above and established as a cell line that responded to G-CSF (LyD9/*fms*-2/GM/G).

Transfection of Human c-fms Gene. The intact human c-*fms* gene in the heavy metal–inducible expression vector and its mutant form(F) in the murine retroviral vector(pZip neoSVX) were kindly provided by Drs. Kato and Sherr (17). Tyrosine at position 996 is replaced with phenylalanine in the mutant form. The product of the mutant c-*fms*(F) is disrupted in a negative regulatory domain near the COOH terminus and is more efficient than its wild-type counterpart in signal transduction. The c-*fms* gene was electroporated into LyD9, L-GM3, and L-G3 with pSV2neo genes, and the selection was started with 1 mg/ml G418 after 48 h. After 2 wk, G418-resistant cells were stained with a mAb against a human c-*fms* product (mAb 3-4A4; Oncogene Science Inc., Manhasset, NY) and subjected to single cell sorting using FACStar® (Becton Dickinson & Co., Mountain View, CA). Cells were expanded with rIL-3 in the presence of G418, 10^{-4} M $ZnSO_4$, and 5×10^{-7} M $CdSO_4$. The c-*fms* (F) gene was transfected into the ϕ2 cells, and transfectants were selected with 1 mg/ml G418. The c-*fms*-expressing ϕ2 cells were sorted by staining with mAb 3-4A4 and expanded for infection. LyD9, L-GM3, and L-G3 were cultured on the monolayer of the c-*fms*-expressing Ψ2 cells for 2 d in the complete medium containing rIL-3 and 5–20 μg/ml of polybrene (Sigma Chemical Co., St. Louis, MO). Cells were harvested and expanded with rIL-3 and G418 for the single cell sorting as described above. All of the c-*fms*-expressing clones were checked for the growth factor dependence and morphology. The experiments were performed with cells keeping the phenotypes of the parent cell lines.

Colony-forming Assays. Colony-forming abilities of LyD9 and LS-1 cells were assayed by their growth in semi-solid agar (0.3%, Bacto-Agar; Difco Laboratories, Detroit, MI) supplemented with growth factors indicated. The results are the average in the triplicate. Colonies are composed of >50 cells and clusters are <50 cells.

Scatchard Plot Analysis. Pure murine rGM-CSF (non-glycosylated) was kindly provided by Kirin Brewery Pharmaceutical Lab. (Gunma, Japan). Iodination of GM-CSF was carried out using the modified two-phase chloramine T method (18). The specific radioactivity of the ^{125}I-labeled GM-CSF was 57,000 cpm/ng, estimated by the self-displacement analysis. Murine rG-CSF was radioiodinated by the IODO-GEN method as described (19). For GM-CSF receptors, cells (3×10^6/tube) were incubated in 200 μl of RPMI 1640 containing 10 mg/ml BSA, 25 mM Hepes (pH 7.4), and various amounts of ^{125}I-GM-CSF at 37°C for 30 min. Each aliquot was layered on a 200-μl mixture of olive oil and dibutyl phthalate (1:4), and centrifuged at 5,000 rpm for 2 min. Cell-

bound(B) and free(F) radioactivity were quantitated with the gamma counter. Nonspecific binding was determined by the addition of a 60-fold molar excess of unlabeled GM-CSF. The binding assay of G-CSF was done as above except that cells (10^7) were incubated at 4°C for 4 h with various concentrations of ^{125}I-G-CSF in 0.3 ml of RPMI 1640 containing 10% FCS and 20 mM Hepes (pH 7.4). A large excess of unlabeled G-CSF was included to determine the nonspecific binding.

Immune Complex Kinase Assay. Cell lysates were prepared from 10^7 cells of transfectants and parent cell lines with 1 ml of RIPA buffer (50 mM Tris HCl, pH 7.4, containing 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate) with 2% aprotinin and 1 mM phenyl-sulphonylfluoride. Cell lysates were precleared with Pansorbin (Calbiochem-Behring Corp., La Jolla, CA) coupled with rabbit anti-rat IgG (Zymed Laboratories, San Francisco, CA). Immune complexes were prepared by the addition of mAb 3-4A4 and incubation at 4°C for 1 h, and then immunoprecipitated with Pansorbin coupled with anti-rat IgG. Precipitates were washed five times with RIPA buffer and then twice with 50 mM Tris HCl (pH 7.4). Kinase reactions were performed as described (12). Products were analyzed in SDS-polyacrylamide gels and detected by autoradiography of the dried gel.

Northern Blot Analysis. Total RNA was isolated by using guanidine isothiocyanate and centrifuging over the cushion of cesium chloride (20). Northern blot analysis was perform using 20 μg RNA as described (21). Blotted filters were hybridized with human c-*fms* cDNA (BamHI fragment) labeled with α-[^{32}P]dCTP (3,000 Ci/mmol; American Corp., Arlington Heights, IL), and rehybridized with human β-actin probe to quantitate RNA.

Morphology. Cells were cytospinned to slide glasses and examined with May-Grüwald-Giemsa stain. Histochemical analysis was performed for myeloperoxidase, naphthol AS-D chloroacetate esterase, and α-naphthylbutyrate esterase using a staining kit (Mutoh Chemicals, Tokyo, Japan). Phagocytosis was determined as described (14).

Results

Expression of Receptors for CSF in LyD9 and Its Derivatives. We first examined expression of receptors for GM-CSF, G-CSF, and M-CSF by the ligand binding assay or by Northern blot analysis. As shown in Fig. 1 *A*, LyD9 and L-G3 cells did not show any specific binding of GM-CSF, whereas L-GM3 cells showed significant specific binding. The Scatchard plot analysis showed that L-GM3 cells expressed ~5,400 GM-CSF receptors per cell with a single K_d value of 80 pM. The results are consistent with previous studies, which stated that L-GM3 cells responded to GM-CSF, resulting in proliferation and differentiation, whereas LyD9 and L-G3 cells did not (9). In contrast, the G-CSF receptor was expressed on not only L-G3 but also LyD9 cells, as shown in Fig. 1 *B*. LyD9 and L-G3 cells expressed ~400 and ~800 receptors, respectively, per cell with the same affinity as that of NFS60 cells from which the G-CSF receptor cDNA was cloned (22). As shown previously L-G3 cells differentiated into neutrophils in response to G-CSF (9). We have not, however, detected any effects of G-CSF on LyD9 cells with or without the addition of IL-3 or IL-4. As expected, L-GM3 cells did not express a detectable level of the G-CSF receptor. NFS60 proliferated without differentiation in the presence of G-CSF.

To assess M-CSF receptor expression, we performed Northern blot analysis of mRNAs using the c-*fms* probe. As shown in Fig. 1 *C*, only an M-CSF-responding cell line, LS-1 (see below), expressed c-*fms* mRNA. Other cells (LyD9, L-GM3, and L-G3), which did not respond to M-CSF, did not express c-*fms* mRNA. Among LyD9 and its derivative cell lines, the expression of GM-CSF and M-CSF receptors is correlated with their response to the growth factors. However, the G-CSF receptor expressed on LyD9 cells could not convey proliferative or differential signals.

LyD9 Cell Line Has Potential to Differentiate into Macrophages. Previous studies showed that LyD9 cells gave rise to myeloid cells as well as B cells when cocultured with primary bone marrow stroma cells (14). The LS series of LyD9 derivatives was established after transferring LyD9 cells cocultured with primary stroma cells onto a cloned stroma cell line, ST2. M-CSF-dependent cells may have been selected because a large amount of M-CSF was secreted from ST2. Morphological characterization of these cells was briefly described previously (14).

As shown in Table 1, one of the LS series clones, LS-1, formed colonies with decreasing frequency in the presence of GM-CSF, IL-3, M-CSF, IL-4, and G-CSF. IL-3 induced compact colonies. GM-CSF induced mainly compact, but some diffuse colonies as well. On the contrary, M-CSF induced only diffuse colonies, as shown in Fig. 2. When LS-1 cells were cultured with M-CSF for 1 wk, Mac-1 expression increased (data not shown), and most cells showed vacuolated cytoplasm. PMA stimulation of LS-1 cells augmented nonspecific phagocytosis. It is interesting to note that there were a small number of neutrophils positive for specific esterase and myeloperoxidase in LS-1 cells cultured in M-CSF for 7–10 d. In the presence of IL-3, the majority of LS-1 cells were immature blast cells, although very few myeloperoxidase-positive neutrophils and nonspecific esterase-positive macrophages were also found.

LyD9 cells could grow only in the presence of IL-3 and formed compact colonies. Although some of cells express Mac-1 and are weakly positive for nonspecific esterase, LyD9 cells did not show any evident signs of differentiation (9, 14). L-GM3 cells respond to either GM-CSF or IL-3 mainly with proliferation, but some myeloperoxidase and specific esterase-positive neutrophils as well as macrophages with phagocytic activities also appeared.

Induction and Expression of the Human c-fms Gene in LyD9 and its Derivatives. To test whether expression of the M-CSF receptor would modify differentiation of LyD9, L-GM3, and L-G3 cells, we introduced the human c-*fms* gene into these cells. We used the wild-type $(Y)^{996}$ and mutant-type $(F)^{996}$ of the c-*fms* gene (12). This replacement of tyrosine with phenylalanine at residue 996 augments M-CSF-dependent response without transformation. Transfected cells obtained were selected with G418 in medium containing IL-3. 2 wk after transfection, G-418-resistant cells obtained were stained with anti-human c-*fms* mAb and subjected to single cell sorting for cloning. Surface expression of the M-CSF receptor on cloned transfectants is shown in Fig. 3 *A*. The remaining clones expressed a similar number of M-CSF receptors.

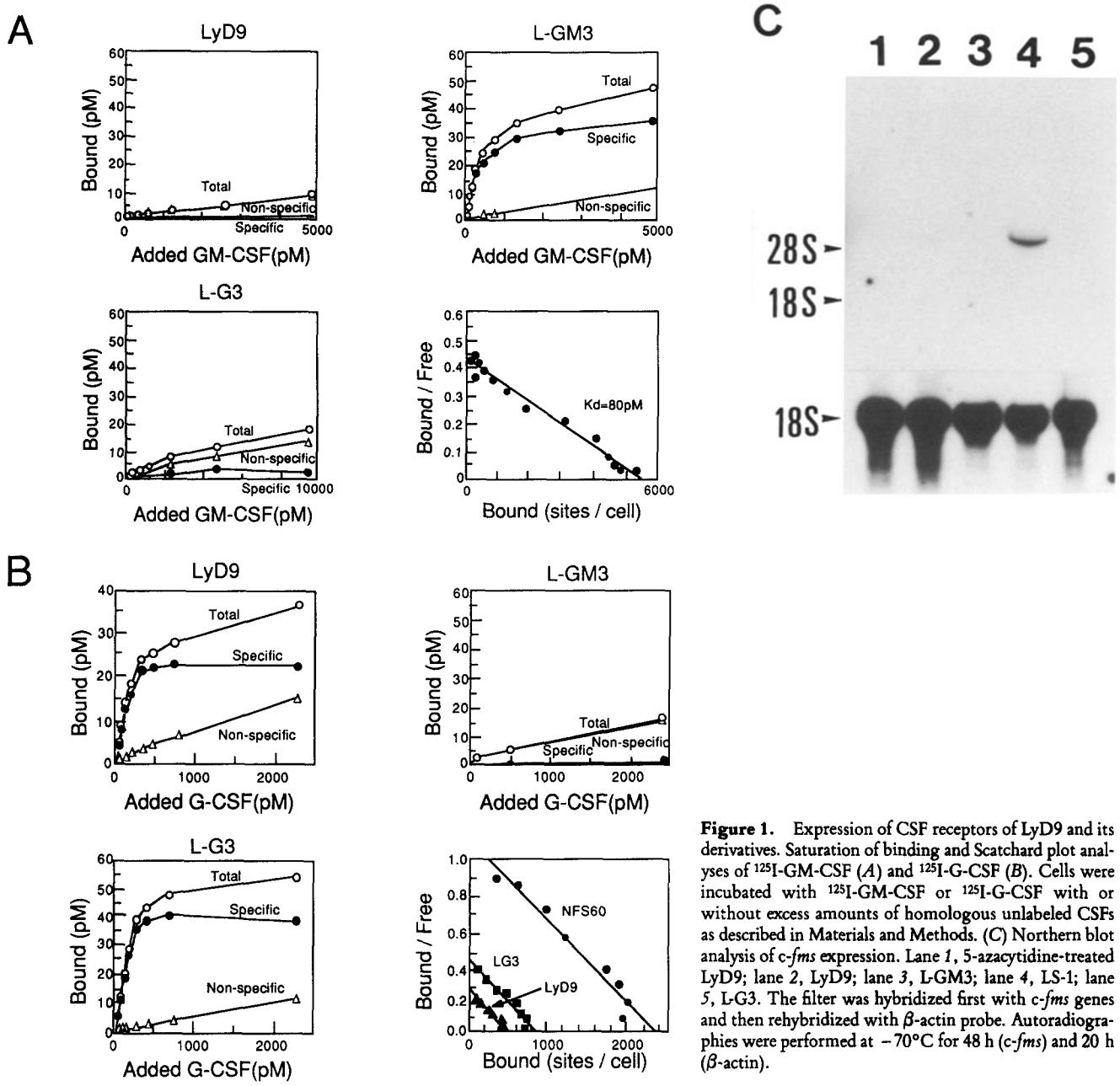

Figure 1. Expression of CSF receptors of LyD9 and its derivatives. Saturation of binding and Scatchard plot analyses of ^{125}I-GM-CSF (*A*) and ^{125}I-G-CSF (*B*). Cells were incubated with ^{125}I-GM-CSF or ^{125}I-G-CSF with or without excess amounts of homologous unlabeled CSFs as described in Materials and Methods. (*C*) Northern blot analysis of c-*fms* expression. Lane *1*, 5-azacytidine-treated LyD9; lane *2*, LyD9; lane *3*, L-GM3; lane *4*, LS-1; lane *5*, L-G3. The filter was hybridized first with c-*fms* genes and then rehybridized with β-actin probe. Autoradiographies were performed at −70°C for 48 h (c-*fms*) and 20 h (β-actin).

Table 1. *Colony-forming Assay of LyD9 and LS-1 Cells*

Cell lines		Growth factors					
		IL-3	IL-4	IL-5	GM-CSF	M-CSF	G-CSF
LyD9	Colonies	67(ND)	0(0)	0(0)	0(0)	0(0)	0(0)
	Clusters	ND(ND)	0(20)	0(0)	0(0)	0(0)	0(0)
LS-1	Colonies	628(ND)	0(880)	0(0)	783(ND)	272(ND)	0(23)
	Clusters	ND(ND)	0(ND)	0(123)	ND(ND)	ND(ND)	ND(217)

Cells (6×10^3 cells) were grown in the soft agar containing IL-3 (100 U/ml), IL-4 (100 U/ml), IL-5 (150 U/ml), GM-CSF (50 U/ml), G-CSF (150 U/ml), or M-CSF (100 U/ml). The results using 6×10^4 cells are shown in parentheses. Colonies (>50 cells) and clusters (<50 cells) were counted on day 7.

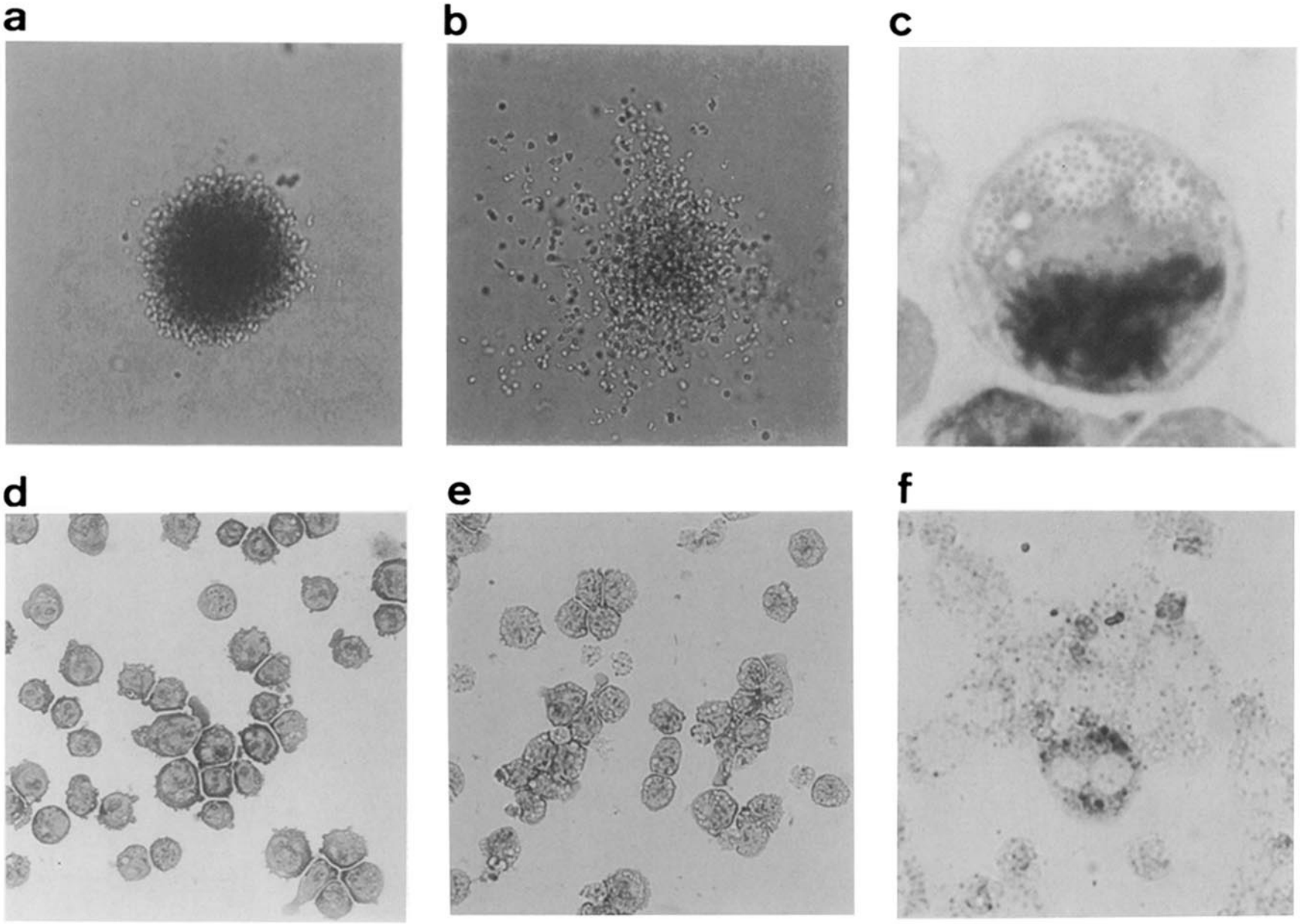

Figure 2. Differentiation of LS-1 with M-CSF. Colony formations of LS-1 cells in a soft agar in the presence of 100 U/ml IL-3 (*a*), and 100 U/ml M-CSF (*b*). Note a compact colony stimulated by IL-3 and a diffuse colony stimulated by M-CSF. (*c*) M-CSF-induced phagocytosis. LS-1 was cultured with 50 U/ml M-CSF for 2 d before the incubation of Latex beeds (Sigma Chemical Co.). Morphology of LS-1 cultured with IL-3 and M-CSF (*e* and *f*) for 7 d, respectively. Cells were stained with May-Grünwald-Geimsa (*d* and *e*) and double stained (*f*) with naphthol AS-D chloroacetate esterase (blue) and α-naphthylbutyrate esterase (brown). Note a specific esterase-positive neutrophil.

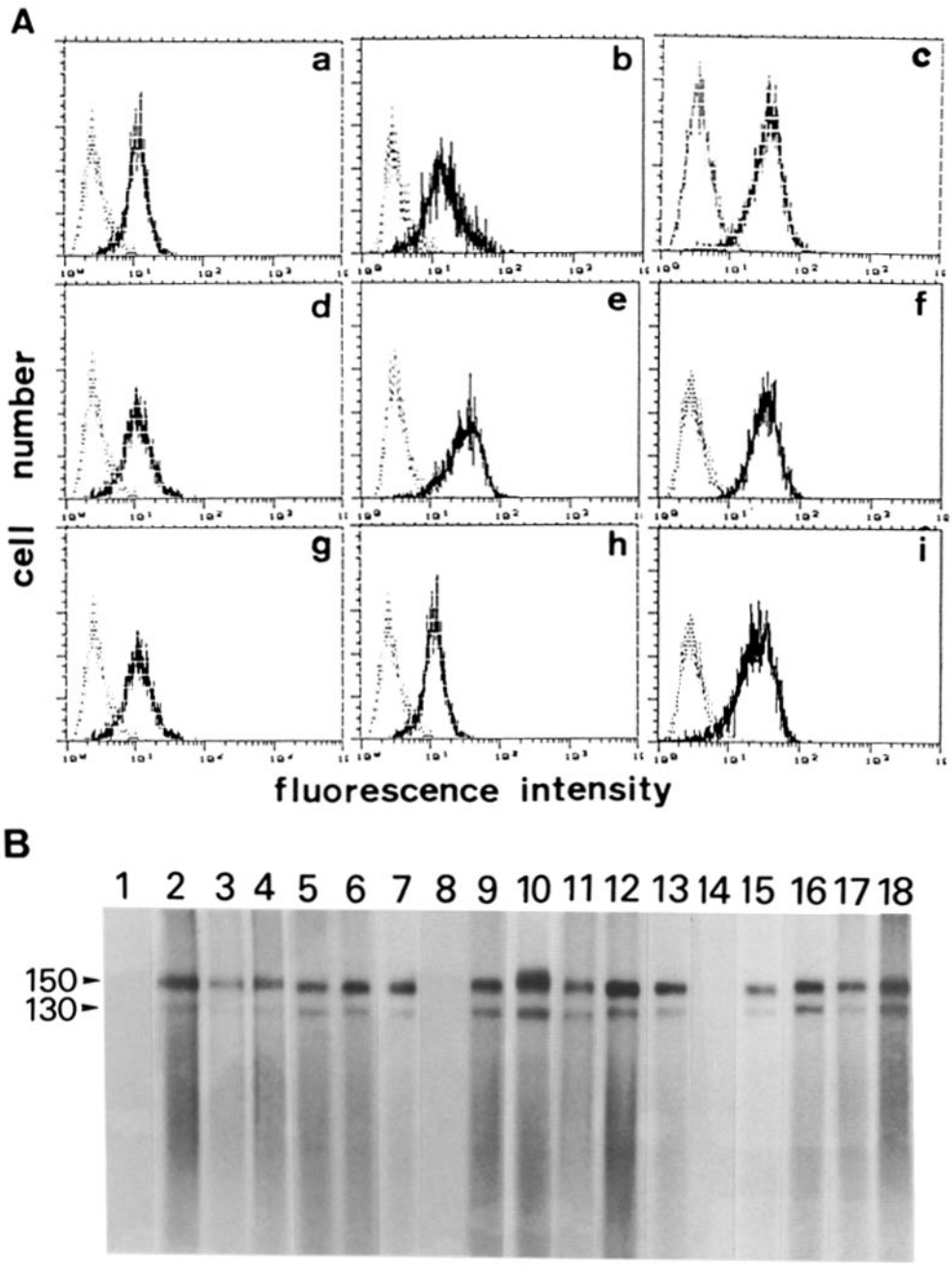

Figure 3. Expression of human c-*fms* product. (*A*) Surface expression of human c-*fms* product of cells transfected with c-*fms* wild (Y) or mutant (F) genes were confirmed with the surface staining with mAb 3-4A followed by FITC-labeled anti-rat IgG as a second antibody. (*a*) LyD9/*fms*-1; (*b*) LyD9/*fms*-2; (*c*) LyD9/*fms*-22; (*d*) L-GM3/*fms*-3; (*e*) L-GM3/*fms*-10; (*f*) L-GM3/*fms*-(F)-3; (*g*) L-G3/*fms*-3; (*h*) L-G3/*fms*-11; (*i*) L-G3/*fms*-(F)-7. Solid lines represent staining patterns with the first and second antibodies, and broken lines represent negative control stained only with second antibody. Patterns of untransfected parent cells stained first with the first and second antibodies were identifical with that of the second antibody only. (*B*) In vitro kinase assay. Kinase activities of expressed human c-*fms* were analyzed with cell lysates prepared from: lane *1*, LyD9; lane *2*, LyD9/*fms*-1; lane *3*, LyD9/*fms*-2; lane *4*, LyD9/*fms*-4; lane *5*, LyD9/*fms*-(F)-2; lane *6*, LyD9/*fms*-(F)-21; lane *7*, LyD9/*fms*-(F)-22; lane *8*, L-GM3; lane *9*, L-GM3/*fms*-3; lane *10*, L-GM3/*fms*-10; lane *11*, L-GM3/*fms*-(F)-1; lane *12*, L-GM3/*fms*-(F)-3; lane *13*, L-GM3/*fms*-(F)-6; lane *14*, L-G3; lane *15*, L-G3/*fms*-3; lane *16*, L-G3/*fms*-11; lane *17*, L-G3/*fms*-(F)-1; lane *18*, L-G3/*fms*-(F)-7. Kinase assay was performed as described in Materials and Methods.

To see whether the expressed c-*fms* proteins were functional, we performed in vitro kinase assay. As shown in Fig. 3 *B*, two phosphorylated proteins were generated in all transfectants. The sizes of phosphoproteins were consistent with those of earlier reports, which demonstrate that the larger band is a mature protein formed by the glycosylation of the smaller band. During the selection with IL-3 and G418, some of the transfectants of L-GM3 and L-G3 cells lost the dependency on GM-CSF and G-CSF, respectively. All experiments for the present study, however, were carried out on transfectants that preserved the growth factor requirement of the parent cells.

Responsiveness of c-fms Transfectants to M-CSF. We then investigated growth responsiveness of the c-*fms* transfectant to human M-CSF. All three LyD9/*fms* clones failed to grow. They survived slightly longer than LyD9 cells, but all cells were dead by day 3 (Fig. 4 *A*). LyD9/*fms*(F) clones showed a transient growth surge for 2 d and declined rapidly. LyD9/*fms*(F) cells showed slightly augmented responses to M-CSF as expected from the observation that the replacement of tyrosine at residue 996 with phenylalanine increases the size and the number of M-CSF colonies of NIH3T3 cells bearing c-*fms* (12). While LS-1 cells displayed M-CSF-dependent growth, it is clear that the M-CSF receptor expressed on LyD9 cells failed to convey signals for continued proliferation.

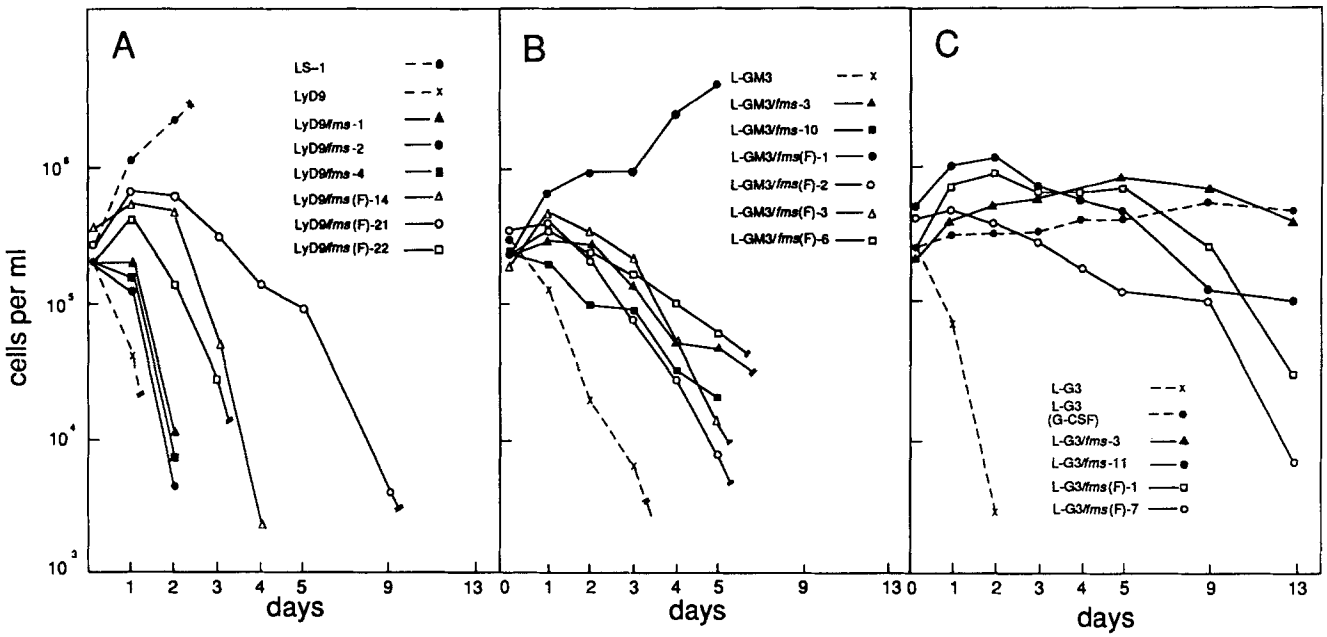

Figure 4. Growth responses to M-CSF. Growth profiles of c-*fms* transfectant of clones LyD9(*A*), L-GM3(*B*), and L-G3(*C*) cells in the presence of M-CSF (50 U/ml). 1–2 × 10^6 cells were grown in 5 ml of the complete medium with or without M-CSF, and the medium was changed with the fresh one every third day. Viable cells were counted with the trypan blue exclusion. LS-1 cells were included as a positive control and untransfected parent cells as a negative control. Growth pattern of c-*fms*-transfected clones without M-CSF were similar to those of parent cell lines. In *C*, growth response of L-G3 cells to G-CSF was included for comparison.

The same pattern was observed for all but one of the L-GM3/*fms* clones (Fig. 4 *B*). L-GM3/*fms*(F) showed a transient response pattern, but declined thereafter. L-GM3/*fms* showed the similar response to a less degree. One clone, L-GM3/*fms*(F)-1, continued to proliferate in the presence of M-CSF. It expressed a similar amount of c-*fms* proteins to other L-GM3/*fms* clones, and its growth is still dependent on M-CSF. We have not characterized any further difference between L-GM3/*fms*(F)-1 and the other clones. As the L-GM/*fms*(F)-1 phenotype is relatively rare, the secondary events might be required for c-*fms* transfectants of LyD9 and L-GM3 cells to acquire the proliferation capability in response to M-CSF.

In contrast, all L-G3/*fms* clones showed prolonged survival in the presence of M-CSF (Fig. 4 *C*). L-G3 transfectants bearing either the wild or mutant type of the M-CSF receptor doubled in number after 24 h and maintained their viability for up to 2 wk. G-CSF induced a similar growth profile except for a longer period up to 18 d.

Differentiation of c-fms Transfectants with M-CSF. We tested whether M-CSF could alter morphological and cytochemical properties of the c-*fms* transfectants. LyD9/*fms* cells showed immature morphology in the presence of M-CSF and did not change this phenotype on day 2, 1 d before death (data not shown). L-GM3/*fms* as well as L-GM3 cells cultured in the presence of either IL-3 or GM-CSF contained a small fraction of neutrophils and macrophages among immature cells (9). Culturing in M-CSF did not change this morphology of the L-GM3/*fms* cells on day 3–4. Most of the L-GM3/*fms*(F)-1 cells, which proliferated continuously in M-CSF, were immature with few mature macrophages and neutrophils (data not shown).

When L-G3/*fms* cells were cultured with G-CSF, these cells began to differentiate on about day 7, and most of the cells became typical mature neutrophils by day 14 (Fig. 5, *c* and *h*). When L-G3/*fms* were cultured in M-CSF-containing media, they showed not only similar growth responses (Fig. 4 *c*), but also similar differentiation profiles (Fig. 5, *d* and *i*) to those induced by G-CSF. Morphological changes became apparent around day 7. Mature neutrophils occupied 20–50% of the population between days 9 and 14. The remaining cells were still immature without monocytic morphological properties such as enlarged and vacuolated cytoplasm, nonspecific esterase, and phagocytic activities, which were typically found in LS-1 cells treated with M-CSF. L-G3 transfectants bearing the mutant type (F) of c-*fms* tended to produce more neutrophils than those bearing the wild-type (Y) (Fig. 5, *e* and *j*). L-G3/*fms* remained immature in IL-3.

Differentiation of LyD9/fms Cells by Stromal Cells. Finally, we tested whether earlier expression of the M-CSF receptor on LyD9 cells would affect the differentiation lineage regulated by cocultured stromal cells (9). LyD9/*fms*-1 and LyD9/*fms*-2 were cultured with PA6 stromal cells in the presence of a minimal amount of IL-3 for 2 wk. Nonadherent cells were harvested and expanded with GM-CSF. Two cell lines, LyD9/*fms*-1/GM and LyD9/*fms*-2/GM, thus established were dependent on either GM-CSF or IL-3. Most of them proliferated without differentiation in the presence of IL-3 or GM-CSF, but some of the cells did differentiate into macrophages and neutrophils (Fig. 5, *a* and *f*). Neither LyD9/*fms*-

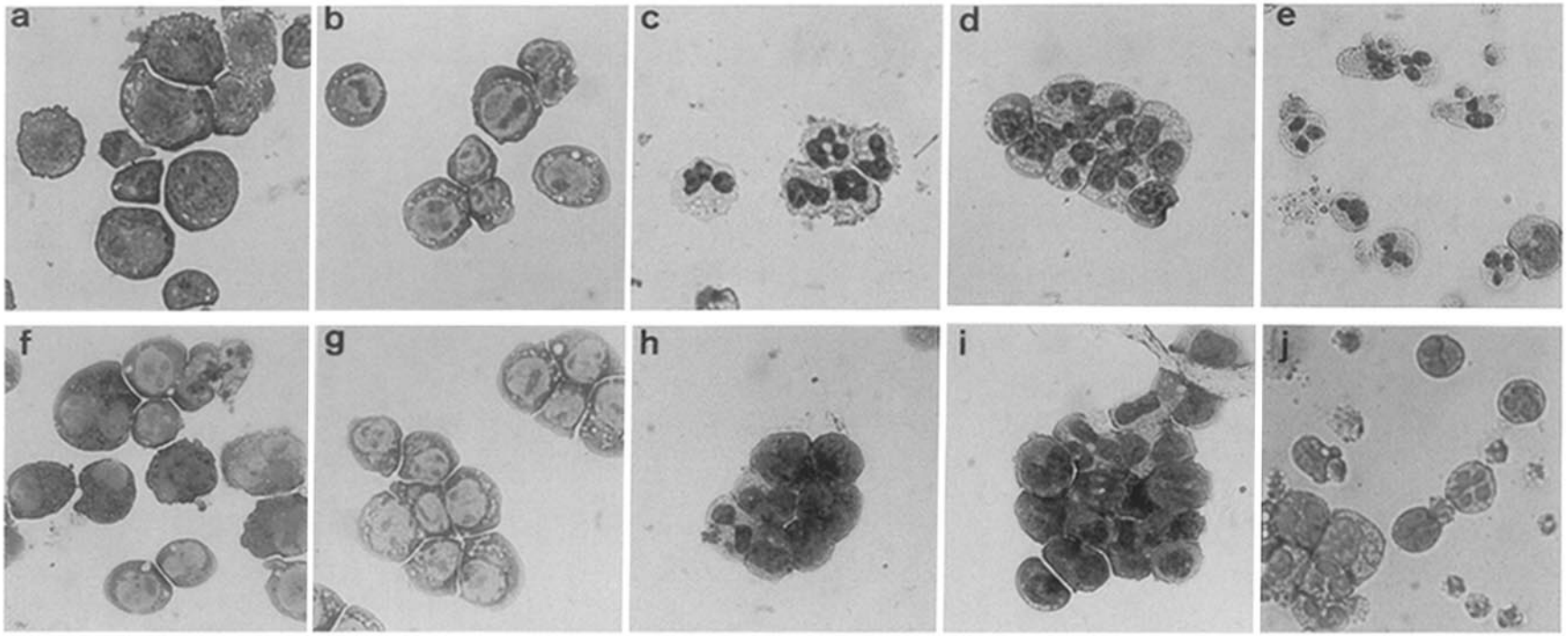

Figure 5. Histological examination of c-*fms*-transfected clones. May-Grünwald-Giesma stain (*a–e*); and myeloperoxidase staining (*f–j*). (*a* and *f*) LyD9/*fms*-1/GM in GM-CSF (10 U/ml); (*b* and *g*) L-G3/*fms*-3 in IL-3 (50 U/ml); (*c* and *h*) L-G3/*fms*-3 in G-CSF (50 U/ml); (*d* and *i*) L-G3*fms*-3 in M-CSF (50 U/ml); (*e* and *j*), L-G3/*fms*-(F)-1 in M-CSF. For the culture with G-CSF and M-CSF, cells were harvested and stained on day 10. L-G3/*fms*-(F)-1 in IL-3 and G-CSF showed similar morphology to those of L-G3/*fms*-3.

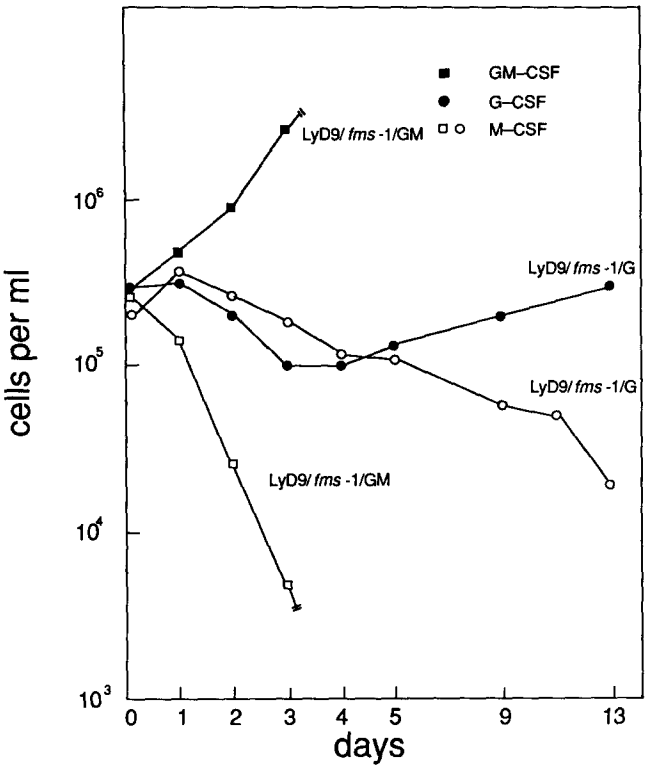

Figure 6. Induction of M-CSF responsiveness after coculture with stromal cells. Growth responses of LyD9/*fms*-1/GM and LyD9/*fms*-1/G cells to GM-CSF (20 U/ml), G-CSF (50 U/ml), and M-CSF (50 U/ml) were measured as described in the legend to Fig. 4.

1/GM nor LyD9/*fms*-2/GM cells were induced to proliferate and differentiate in response to human M-CSF (Fig. 6). No cell lines were obtained even when nonadherent cells were cultured in human M-CSF. Expression of the M-CSF receptor on LyD9/*fms*-1 (or -2)/GM cells was confirmed by surface staining with the anti-human c-*fms* antibody (data not shown).

LyD9/*fms*-1 cells were cocultured with ST2 stromal cells to induce G-CSF-dependent lines. After 2 wk of coculture, nonadherent cells were harvested and maintained with G-CSF for 2 d, and then expanded and maintained with IL-3. The growth response of this line (LyD9/*fms*-1/G) to G-CSF and M-CSF is shown in Fig. 6. LyD9/*fms*-1/G responded to both G-CSF and M-CSF with the same prolonged survival pattern as found in L-G3/*fms* cells. LyD9/*fms*-1/G cells survived for ~18 d in G-CSF but for ~12 d in M-CSF. Morphological studies showed that most of the LyD9/*fms*-1/G cells differentiated into neutrophils in the presence of G-CSF, whereas 30–40% of cells were neutrophils in the presence of M-CSF. Identification of neutrophils was based on ringed or segmented nuclei, and myeloperoxidase production (Fig. 7). None of LyD9/*fms*-1/G cells cultured in M-CSF showed any characteristics of macrophages such as phagocytosis and expression of nonspecific esterase (data not shown). Similar lines were obtained when M-CSF was added in place of G-CSF for 2 d before maintaining the cells in IL-3.

Similar differentiation induction experiments using ST2 stroma cells were done with L-GM3/*fms* and LyD9/*fms*/GM cells. G-CSF-responsive derivative lines of L-GM3/*fms* and LyD9/*fms*/GM cells were obtained by culturing for 2 d in G-CSF-containing media and subsequent expansion with IL-3 after coculture with ST2 cells. When these derivatives were further cultured with either G-CSF or M-CSF, they differentiated into neutrophils but not macrophages in a similar manner, as mentioned above (Fig. 7). When the cells cocultured with ST2 stromal cells were transferred directly to M-CSF-containing media, they also differentiated into neutrophils but not macrophages (data not shown). These results indicate that premature expression of the M-CSF receptor does not direct the monocytic commitment of LyD9, L-GM, and LG3 clones, nor does it alter particular differentiation lineage induced by coculture with specific stromal cells.

Discussion

We have tested whether forced expression of the M-CSF receptor in a multipotential stem cell line and myeloid progenitor cells would affect determination of differentiation lineage. The multipotential cell line LyD9 proliferates without differentiation in response to IL-3 and has been shown to differentiate into neutrophils, macrophages, and B lymphocytes by coculture with different stromal cells (9, 14, 23–25). L-GM3 and L-G3 cells derived from LyD9 cells proliferate and differentiate in response to GM-CSF and G-CSF, respectively (9). We found that the M-CSF receptor expression in these stem and progenitor cells and progenitor cells did not induce differentiation into macrophages in the presence of M-CSF. Instead, c-*fms* transfectants of L-G3 cells differentiated into neutrophils in response to M-CSF. The results clearly indicate that the M-CSF receptor requires a signal transduction system different from those of the IL-3, GM-CSF, and G-CSF receptors. Furthermore, the signal transduction pathway of the M-CSF receptor seems to cooperate with that of the G-CSF receptor.

We have also tested whether the premature expression of the M-CSF receptor in the multipotential stem and progenitor cells would affect the direction of differentiation induced by coculture with stromal cells. We found that the myeloid lineage of differentiation was controlled primarily by the type of stromal cells used for coculture regardless of the prior expression of the M-CSF receptor in the stem and progenitor cells.

The differentiation induction through a growth factor receptor depends on expression of at least three sets of gene products whose functions are tightly linked: (*a*) the growth factor receptor, (*b*) cytoplasmic signal transduction machinery, and (*c*) transcriptional machinery that makes target genes ready for induction. In other words, the growth factor receptor cannot induce proliferative or differentiational effects if any of the components in categories *b* and *c* is not expressed in progenitor cells. The c-*fms* transfectants of LyD9, L-GM3, and L-G3 cells were unable to transduce the M-CSF-specific signals even though they express the M-CSF receptor that can catalyze phosphorylation. It is not clear which of cytoplasmic signal transduction and transcriptional machineries specific to the M-CSF receptor is missing in these cells. Although the c-*fms* transfectants of the L-G3 and other G-CSF-

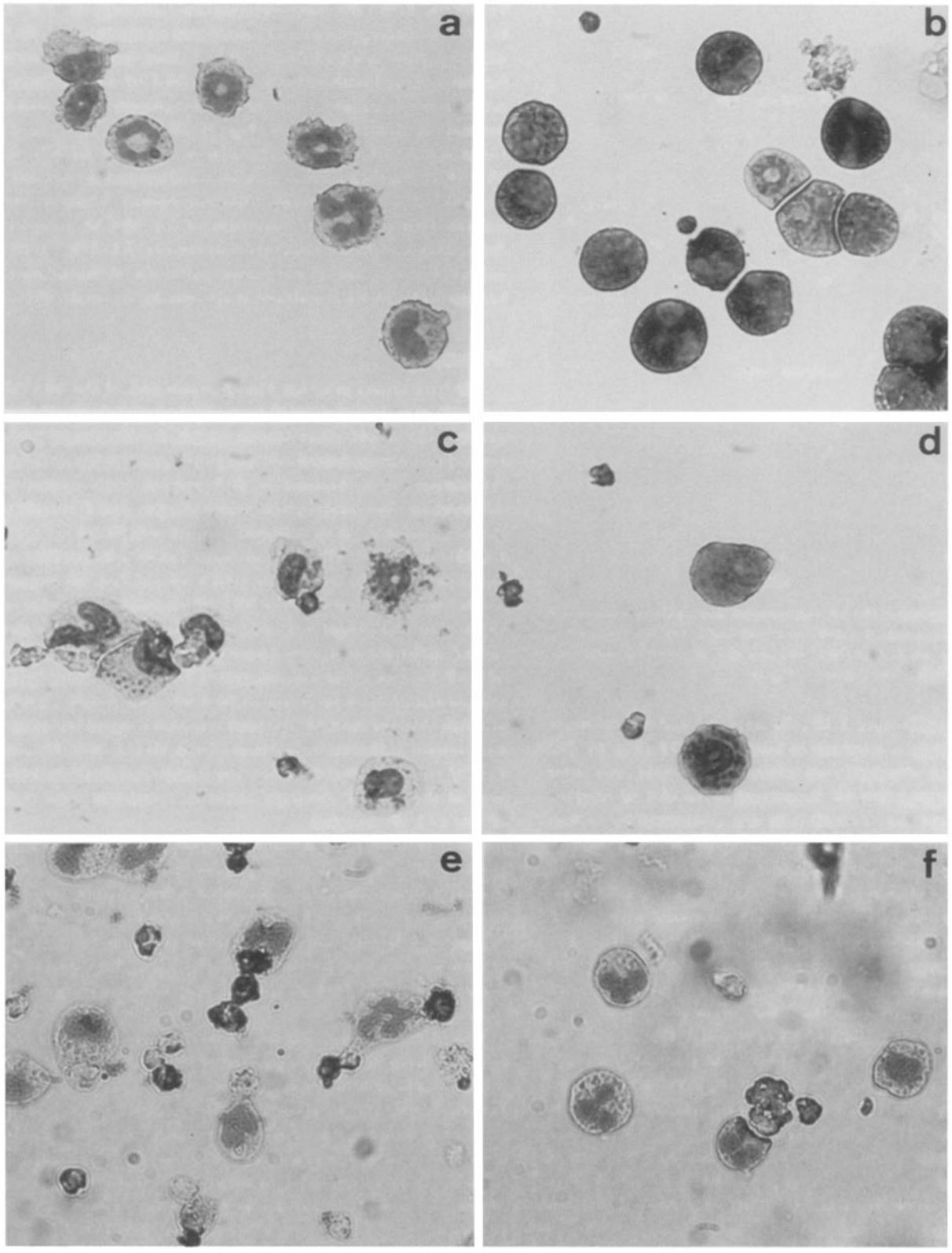

Figure 7. M-CSF- and G-CSF-induced differentiation of c-*fms* transfectants cocultured with ST-2 stromal cells. LyD9/*fms*-1G (*a–d*) were with May-Grünwald-Giemsa (*a* and *c*) and myeloperoxidase (*b* and *d*) after culturing with 50 U/ml G-CSF (*a* and *b*), or 50 U/ml M-CSF (*b* Cells derived from LyD9/*fms*-2/GM cocultured with ST2 stromal cells were cultured with 50 U/ml G-CSF (See Materials and Methods) (U/ml M-CSF (*f*), and stained with May-Grünwald-Giemsa.

responsive cell lines derived from LyD9 cells differentiated into granulocytes in response to M-CSF, it is not clear where the signal from the M-CSF receptor crossed the G-CSF-specific pathway. This crossing can take place at either the cytoplasmic signal transduction machinery or the selection of target genes. Since a high concentration of M-CSF has been shown to stimulate neutrophil development, as shown in LS-1 and other cells (26), the differentiation induction machineries of M-CSF and G-CSF appear to cross or share some components.

It has been long debated whether the interaction of the growth factor and its receptor is deterministic to lineage commitment. As mentioned above, the expression of the growth factor receptor alone can not be deterministic. The growth factor may appear to determine lineage commitment when the whole differentiation induction system, including the receptor, cytoplasmic signal transduction machinery, and transcriptional machinery, is ready in progenitor cells. However, one can argue that the cell at this stage has been already committed to the lineage to be induced by the growth factor. Choice of a single cell lineage from several potentials is likely to involve a series of biochemical events as described above. In this sense, lineage commitment may be a continuous process until the genes involved in the growth factor differentiation induction are all expressed. Only when a progenitor cell has established differentiation induction systems for multiple growth factors can one of the growth factors be deterministic (27). It is important to know how the differentiation induction system for a lineage-specific growth factor is activated in the progenitor cell.

Our accompanying study (9) indicates that coculture with different stromal lines support differentiation of the LyD9 stem cell line into specific lineages. It has been shown that direct contact between LyD9 cells and stromal lines is essential for differentiation. We have now shown that the prior expression of the M-CSF receptor does not alter the differentiation program controlled by the stromal cell lines. Although LyD9 cells were induced to differentiate into macrophages by coculture with heterogenous primary stromal cells, ST2 and PA6 stromal lines could not replace them for macrophage differentiation of LyD9 cells. As both ST2 and PA6 stromal cells produce murine M-CSF, their inability to support macrophage differentiation should be explained by the absence of either unknown growth factors produced by stromal cells, interaction through cell adhesion molecules, or their combination.

Other groups of investigators have shown that transfected c-*fms* is able to transduce proliferative and/or differentiation signals in myeloid and pre-B cell lines. Human c-*fms*-transfected 32D cells, which are G-CSF responsive and similar to L-G3 cells, were able to proliferate continuously and to differentiate reversibly to macrophages in response to M-CSF (28). These results are in a sharp contrast to our own but are not necessarily contradictory. The two cell lines must differ in the expression of component of the M-CSF differentiation induction system. It is not surprising that there are multiple intermediate stages of expression of components of the differentiation induction system for lineage-specific growth factors. c-*fms* transfectants of another myeloid progenitor line, FDC-P1, which is similar to the L-GM3 cells, can proliferate continuously in the presence of M-CSF (17). A similar study using murine c-*fms* showed c-*fms* transfectants of FDC-P1 cells proliferate and differentiated reversibly into macrophages in response to murine M-CSF (29). FDC-P1 and 32D cells appear to contain necessary components of the M-CSF differentiation induction system.

A c-*fms*-transfected clone (DIF9) of IL-7-responding cells was isolated by selection in lymphocyte-growing media with M-CSF. DIF9 cells could proliferate and differentiate irreversibly into macrophages by shifting to myeloid-growing media with M-CSF (30). Although this study has concluded that signals mediated by the transfected M-CSF receptor play a deterministic role in cell differentiation, it remains to be seen whether this observation is general and physiological because of the following reasons. (*a*) The lineage switching requires not only M-CSF but also myeloid-growing media. In fact, DIF9 cells kept pre-B phenotype in RPMI 1640 containing M-CSF. (*b*) To claim the lineage switching, it is important to show that DIF9 cells retain the maturation potential to B cells. Although DIF9 cells proliferate in response to IL-7 and have rearranged the Ig heavy chain loci, DIF9 cells do not express the μ chain protein. DIF9 cells could be in a dead end of differentiation into B lymphocytes. (*c*) Only a single clone of c-*fms* transfectants showed the "lineage switching" phenotype. It must be a very rare event even if it can happen.

In addition to difference in expression of the M-CSF differentiation induction system, experiments described above may contain in vitro artifacts that can arise from using M-CSF during selection of c-*fms* transfectants. We are careful to avoid such artifacts and always tested both mixed populations and independent clones. In fact, we also isolated one L-GM3/c-*fms* clone that proliferated continuously in response to M-CSF. It may be possible therefore that relatively rare M-CSF-responsive cells could only be selected by culturing in M-CSF-containing media (30, 31).

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