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Macrophage Lineage Switching of Murine Early Pre-B Lymphoid Cells Expressing Transduced *fms* Genes

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***fms* genes encoding either wild-type or constitutively activated colony-stimulating factor 1 receptors (CSF-1R) were introduced by retroviral infection into long-term mouse lymphoid cultures. Four early pre-B-cell lines transformed by the feline v-*fms* oncogene underwent spontaneous and irreversible differentiation to macrophages when transferred from RPMI 1640 to Iscove modified Dulbecco medium. Expression of wild-type human CSF-1R in early pre-B cells conferred no proliferative advantage unless human CSF-1 was added to the culture medium. A clonal, factor-dependent early pre-B-cell line (D1F9), selected for continuous growth on NIH 3T3 cell feeder layers producing human CSF-1, could be maintained in RPMI 1640 medium containing interleukin-7 (IL-7) but also differentiated to macrophages when grown in Iscove modified Dulbecco medium containing human CSF-1. The macrophages retained parental immunoglobulin gene rearrangements and proviral insertions, lost B-cell antigens, expressed butyrate esterase and MAC-1, were actively phagocytic, and no longer survived in IL-7. Unlike factor-independent v-*fms* transformants, the irreversible commitment of D1F9 cells to differentiate in the macrophage lineage could be suppressed by IL-7, depended on human (but not mouse) CSF-1, and was inhibited by an antibody to human CSF-1R. Signals mediated by transduced CSF-1R can therefore play a deterministic role in cell differentiation.**

Colony-stimulating factor 1 (CSF-1 or M-CSF) is a lineage-specific growth factor that supports the proliferation, differentiation, and survival of cells of the mononuclear phagocyte lineage (reviewed in references 53a and 55). It differs from other pluripoietins, such as interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which act through their own receptors to stimulate immature bone marrow precursors capable of differentiating to macrophages (reviewed in reference 36). The effects of CSF-1 are mediated through its binding to a cell surface receptor (CSF-1R) encoded by the c-*fms* proto-oncogene (53). CSF-1R exhibits a ligand-dependent protein tyrosine kinase activity (42, 53, 62) and is most closely related to the A- and B-type receptors for platelet-derived growth factor (11, 33, 60) and to the c-*kit* proto-oncogene product, a putative receptor for an as yet uncharacterized ligand (41, 61). The pleiotropic effects of CSF-1 are due to the receptor-mediated phosphorylation of intracellular proteins, some of which act to relay signals that regulate CSF-1-responsive genes (reviewed in reference 51).

Because expression of CSF-1R is a hallmark of maturation within the mononuclear phagocyte lineage (10, 19), it has been difficult to evaluate whether receptor-mediated signals play any deterministic role in macrophage differentiation or rather serve only to support lineage-specific cell maturation and survival. CSF-1 induces only macrophage colonies from bone marrow cells cultured in vitro (56), suggesting that it predominantly stimulates monopotent progenitors. Although it can act synergistically with IL-3 and GM-CSF to stimulate macrophage colony formation from more primitive progenitors than those responsive to CSF-1 alone (3, 26, 30, 37, 54), IL-3 or GM-CSF can themselves induce the development of macrophage colonies by supporting a stochastic process of commitment to single lineages (23, 25, 35, 57). High GM-CSF concentrations alone favor granulocyte colony development, whereas low concentrations preferentially induce macrophage colonies (35, 37). However, if bipotential myeloid progenitors are first stimulated with CSF-1 to undergo several divisions, only macrophage colonies develop, even if high concentrations of GM-CSF are substituted as a continuing stimulus (27). CSF-1 can therefore foster the formation of macrophage progeny from multipotential cells, and the process of commitment appears to entail a loss of responsiveness to other hemopoietins.

One approach for studying the effects of CSF-1 on cell differentiation has been to insert the c-*fms* gene into established mouse myeloid cell lines that are dependent on IL-3 or GM-CSF for their growth and survival in vitro. Such cells can be reprogrammed to proliferate in CSF-1, and in some cases (46) but not others (28), phenotypic markers characteristic of myeloid differentiation were induced by CSF-1 stimulation. However, only partial differentiation toward macrophages was observed, and the process was reversed when the cells were retransferred to medium containing IL-3 or GM-CSF (46).

The v-*fms* oncogene encodes a mutated form of CSF-1R that functions constitutively as a kinase in the absence of CSF-1 (48, 49, 59). Infection of mouse long-term lymphoid cultures with a v-*fms*-containing retrovirus resulted in the rapid clonal outgrowth of early pre-B cells which initially depended on stromal-cell feeder layers for proliferation but eventually gave rise to factor-independent variants (7). We reasoned that insertion of the human c-*fms* gene into mouse lymphoid cells might provide a means of developing early pre-B-cell lines that would be conditionally dependent upon exogenous human recombinant CSF-1 for their continuous propagation in vitro. In addition, the fact that transformation of early B-cell progenitors with different oncogenes can immortalize biphenotypic B-cell progenitors capable of spontaneous macrophage differentiation (8, 14, 21, 29) raised

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the possibility that transduced *fms* genes might enable a differentiative response. We now describe the properties of a c-*fms*-positive early pre-B-cell line (D1F9) which is able to proliferate in either mouse IL-7 or human CSF-1. These cells, as well as four previously established early pre-B-cell lines transformed by v-*fms*, differentiate to functional macrophages when transferred into medium that preferentially supports the growth of myeloid cells. Lineage switching of D1F9 cells depended upon the presence and activity of transduced human CSF-1R, implying that CSF-1 can directly affect the expression of genes that program macrophage development.

MATERIALS AND METHODS

Culture conditions and viral infection. Bone marrow cells from the femurs and tibias of BALB/c mice (Charles River Breeding Laboratories, Wilmington, Mass.) were washed and suspended at 10^6 cells per ml in lymphoid culture medium (RPMI 1640, 5% fetal calf serum [FCS], 10 mM glutamine, 5×10^{-5} M 2-mercaptoethanol [2-ME]), and 5-ml cultures in 60-mm-diameter dishes grown at 37°C in 6% CO_2 were fed biweekly, as originally described by Whitlock and Witte (58). Different lots of FCS (GIBCO Laboratories, Grand Island, N.Y.) were prescreened for their ability to support B lymphoid cell growth as compared with preselected samples generously provided by Judy Young and Owen Witte (Howard Hughes Medical Institute, University of California School of Medicine, Los Angeles, Calif.).

Retroviral vectors containing either wild-type or mutant (969F) human c-*fms* genes (12, 49) were transfected into the ψ2 packaging cell line (32), and virus-producing cells, selected in medium containing 800 μg of G418 (Geneticin; Sigma Chemical Co., St. Louis, Mo.) per ml, were purified by fluorescence-activated cell sorting by using monoclonal antibodies to human CSF-1R (2) and recloned from single cells in microdilution culture plates. Viruses produced from subcloned cell lines were assayed for their ability to produce foci by an autocrine mechanism after titration on NIH 3T3 cells producing human recombinant CSF-1 (20, 49). Subclones producing high-titer *fms* viruses ($>3 \times 10^5$ focus-forming and neomycin-resistance U/ml) were identified, and 75% of the medium in long-term bone marrow cultures was removed and replaced by filtered supernatants from cultures producing helper-free viruses. All infections were performed 5 weeks after establishment of the B lymphoid cultures, at a time when confluent stromal feeder layers had formed and when active lymphopoiesis had begun.

NIH 3T3 cells engineered to produce membrane-bound (44) or secreted (43) forms of human recombinant CSF-1 were derived previously. In some experiments, normal NIH 3T3 cells or their CSF-1-producing subclones were used as feeder layers, either as artificial stroma or as supporting monolayers for colony formation in Iscove modified Dulbecco medium (IMDM) containing 0.3% Noble agar. Cells cloned by endpoint dilution in microdilution wells were expanded and shifted into myeloid culture medium (IMDM containing 5% FCS, 5×10^{-5} M 2-ME, 10^{-7} M hydrocortisone, vitamins, and amino acids [GIBCO, Grand Island, N.Y.] as originally described by Dexter and co-workers [15, 16]). Although hydrocortisone is included to suppress lymphopoiesis in mouse long-term myeloid cultures, all *fms*-infected cell lines grown in myeloid culture medium were found to be steroid-resistant by the time of transfer to myeloid medium.

Other analytical methods. High-molecular-weight DNA was analyzed by Southern blotting on Nytran filters (Schleicher & Schuell, Inc., Keene, N.H.) as previously described (6). Probes radiolabeled by nick translation and relevant restriction maps were identical to those used previously (7) (see Fig. 3). Preparation of cell lysates, immunoprecipitation, immune complex kinase assays, and conditions for electrophoresis in polyacrylamide gels were previously described (1, 50). Procedures for fluorescence-activated cell sorting and histochemical staining (2) have also been described in detail. In situ hybridization with an antisense c-*fms* riboprobe was performed as described by Lawrence and Singer (31). A 3.1-kilobase *Bam*HI fragment of the human c-*fms* gene was subcloned into the pGEM-1 plasmid (Promega Biotec, Madison, Wis.). Antisense probes were generated with SP6 RNA polymerase by using *Eco*RI-linearized plasmids. Conversely, control sense probes were generated with T_7 RNA polymerase on *Hin*dIII-linearized plasmids.

To determine whether both human and murine CSF-1R were coexpressed in cell lines undergoing macrophage differentiation, cell lysates were immunoprecipitated with a rat monoclonal antibody (MAb) specific for human CSF-1R (2), and the centrifuged supernatants were reprecipitated with a rabbit antiserum cross-reactive with the murine receptor (17). Washed immunoprecipitates were incubated in an immune complex kinase assay with [γ-^{32}P]ATP for 10 min at room temperature, and the labeled products were separated on denaturing polyacrylamide gels and detected by autoradiography (50).

Special reagents. Human recombinant CSF-1 was provided by Steven Clark (Genetics Institute, Cambridge, Mass.) and IL-7 was provided by Judy Young and Owen Witte (Howard Hughes Medical Institute, UCLA School of Medicine, Los Angeles, Calif.). The source of IL-7 was conditioned medium from a transfected COS-7 cell line. One unit of IL-7 (formerly termed lymphopoietin 1) is defined as the amount of factor required to induce half-maximal [^{3}H]thymidine incorporation in a stimulation assay involving 1.25×10^4 pre-B cells (40). Human c-*fms* cDNA (12) was originally provided by Axel Ullrich (formerly from Genentech, South San Francisco, Calif.), and human 1.6- and 4-kilobase CSF-1 cDNAs encoding membrane-bound and soluble forms of the growth factor, respectively, were from Peter Ralph (Cetus Corporation, South San Francisco, Calif.). The pZIP*neo* SVX(I) vector and ψ2 cells were provided by Richard Mulligan (Whitehead Institute, Cambridge, Mass.). MAbs to human CSF-1R (2, 52) are available for investigational use through Oncogene Sciences, Manhasset, N.Y.

RESULTS

Establishment of CSF-1-responsive early pre-B-cell lines. Long-term murine bone marrow cultures able to support the growth of B lymphoid cells consist of an underlying stromal feeder layer upon which active hematopoiesis occurs (58). The adherent feeder layer is composed primarily of fibroblasts, macrophages, and endothelial cells, and produces a soluble growth-promoting activity that includes IL-7 (24, 39, 40). Five weeks after establishment, when active lymphopoiesis had been initiated, the cells were infected with filtered culture supernatants containing helper-free retroviruses into which human c-*fms* cDNAs had been inserted (49). We used two different constructs containing either the wild-type c-*fms* gene or a mutant in which a tyrosine residue in the distal carboxyl-terminal tail of CSF-1R had been converted to a phenylalanine residue. CSF-1R(969F) does

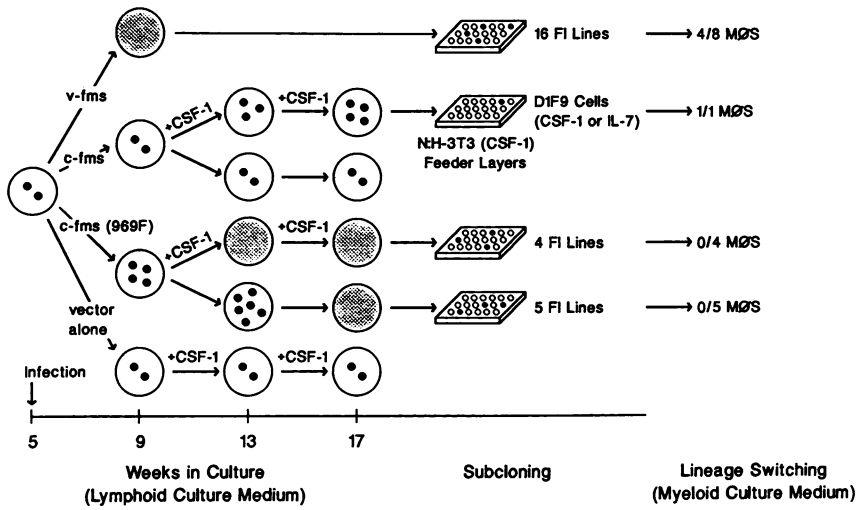

FIG. 1. Derivation of murine long-term cultures infected with *fms*-containing retroviruses. Cultures (large circles) were infected with retroviruses containing v-*fms*, wild-type c-*fms*, or mutant c-*fms*(969F) genes 5 weeks after establishment. Small filled circles denote the relative density of lymphoid cell growth, whereas hatched cultures signify those in which lymphoid cells were induced to proliferate to unusually high densities (>1.5 × 10^6/ml). From 10 to 30 separate cultures were infected with each retrovirus. Lymphoid growth medium collected from NIH 3T3 cells producing human recombinant CSF-1 (2 nM, final concentration) was added to half of the cultures at week 9. Factor-independent v-*fms*- and c-*fms*(969F)-infected cells were cloned by limiting dilution in microdilution wells in lymphoid growth medium alone. The factor-dependent D1F9 cell line was cloned by similar methods on a surrogate stroma of NIH 3T3 cells expressing a membrane-bound form of human CSF-1. The numbers of cultures tested for lineage switching are indicated at the right.

not transform mouse NIH 3T3 cells but mediates an enhanced growth-promoting response to ligand (28, 49). Control lymphoid cell cultures were infected with the retroviral vector alone. Factor-independent (FI) early pre-B-cell lines transformed by the v-*fms* oncogene were derived previously (7).

As summarized schematically in Fig. 1, cells expressing the v-*fms* gene proliferated to abnormally high densities within 1 week of infection and three weeks later contained 20- to 30-fold-more nonadherent cells (2.5 × 10^6/ml) than did uninfected cultures (1 × 10^5/ml), necessitating their biweekly demidepopulation. These cells subsequently yielded FI variants that could be maintained indefinitely in lymphoid growth medium without feeder layers (RPMI 1640 with 2-ME, glutamine, and 5% FCS). At 12 weeks postinfection, each independently derived v-*fms*-infected culture consisted of a few dominant early pre-B-cell clones, as determined by restriction enzyme analyses of immunoglobulin heavy-chain gene rearrangements and sites of proviral insertion (7; see also Fig. 3, lanes 1 through 5). Sixteen such cultures were recloned by endpoint dilution in microdilution wells, each yielding FI subclones derived from single cells.

Cells infected with the wild-type human c-*fms* gene proliferated at the same rate as uninfected or vector-infected cultures, but those infected with c-*fms*(969F) grew more rapidly. Four weeks after infection, human recombinant CSF-1 was added to half of these cultures. Although the stromal cells in long-term bone marrow cultures produce murine CSF-1, the mouse growth factor does not bind with high affinity or stimulate the growth of cells expressing human CSF-1R (13, 48, 49). By 8 weeks after infection (Fig. 1, week 13), cells infected with the wild-type human c-*fms* gene grew to a somewhat higher density in response to CSF-1 than did uninfected cells (Fig. 2A and B), but those infected with c-*fms*(969F) now showed an enhanced proliferative response (Fig. 2C). Human CSF-1 had no effect on uninfected cells or on cells infected with the vector alone. In situ hybridization performed at week 13 with a c-*fms* antisense riboprobe confirmed that a high proportion of the cells in cultures infected with the wild-type gene expressed c-*fms* RNA (Fig. 2E), whereas those infected with c-*fms*(969F) were uniformly positive (Fig. 2F). In agreement, fluorescence-activated cell sorting analysis performed with an antibody to human CSF-1R demonstrated receptor epitopes on all infected populations, and detergent lysates from representative cultures contained immunoprecipitable CSF-1R tyrosine kinase activity (data not shown). The cells were small and round and lacked substantial cytoplasm, consistent with a lymphoid morphology (Fig. 2A through F).

The ability of the c-*fms* genes to transduce a CSF-1-dependent growth response in lymphoid cells was tested by seeding previously unexposed infected cells from week 13 cultures onto NIH 3T3 monolayers expressing either membrane-bound or soluble forms of CSF-1 (43, 44). Three days after plating, small clusters of round cells were observed on the artificial stroma, and by 14 days, the clusters had grown into large, refractile colonies (Fig. 2H and I). In contrast, lymphoid cells transferred from cultures infected with the vector alone were unable to form colonies on these feeder layers (Fig. 2G). Table 1 shows that the frequency of colonies obtained with cells expressing CSF-1R(969F) were about twofold higher than those expressing wild-type CSF-1R. Cells expressing the wild-type receptor could not form colonies on normal NIH 3T3 monolayers, whereas a small percentage (0.03%) of cells expressing CSF-1R(969F) produced colonies in the absence of CSF-1. X-irradiated monolayers producing the membrane-bound form of the growth factor were also able to support colony formation of CSF-1R-positive cells (Table 1), but glutaraldehyde-fixed monolayers were not. Vigorous washing of the monolayers released colony-forming cells which stained with a fluorescent antibody directed to human CSF-1R (data not shown). Thus, both wild-type and mutant CSF-1R could render lymphoid cells responsive to human CSF-1.

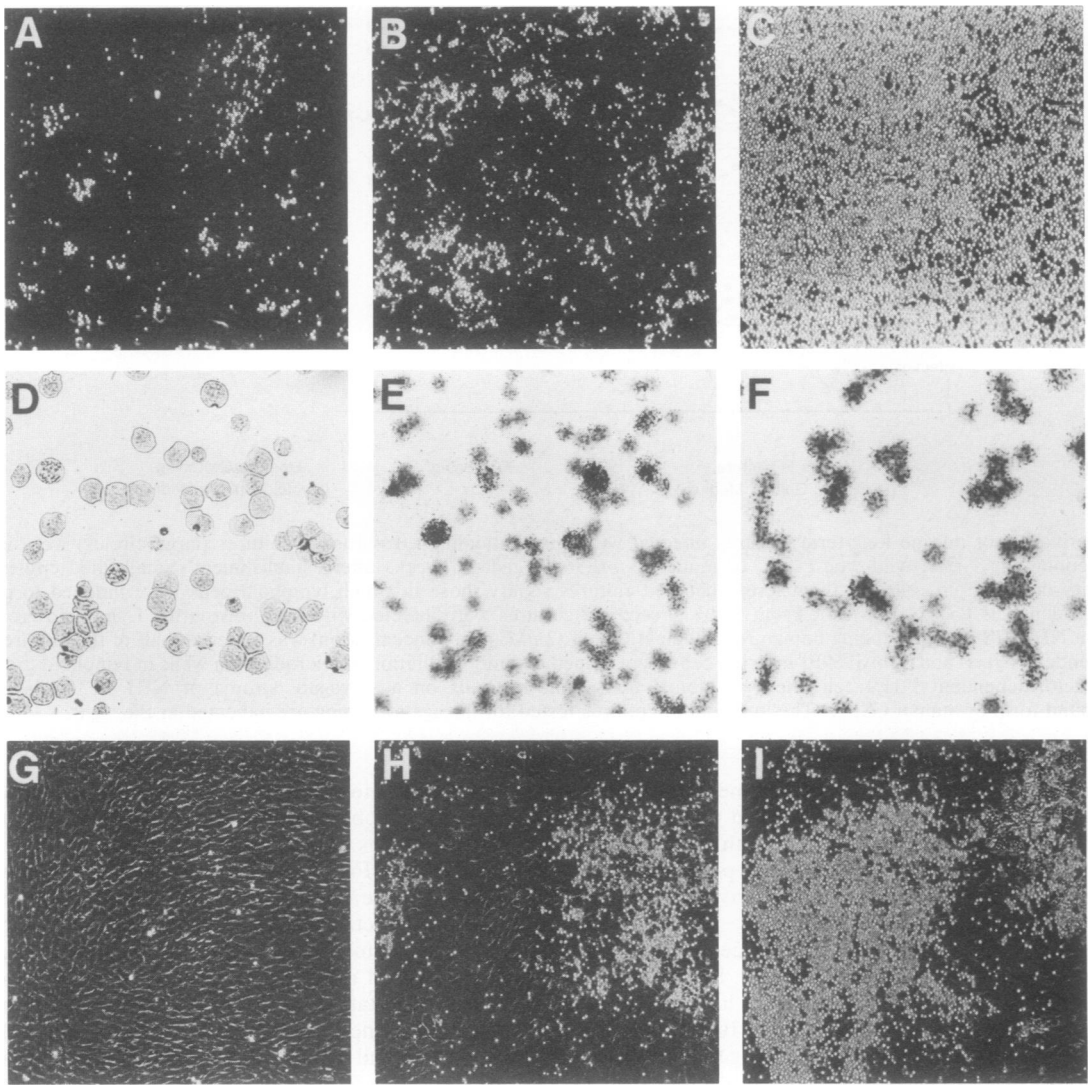

FIG. 2. Growth properties of lymphoid cultures infected with *fms*-containing retroviruses. The top panels show phase-contrast micrographs of lymphoid cultures infected with vector alone (A), c-*fms* (B), or c-*fms*(969F) (C), grown in the presence of human CSF-1. Cells infected with the respective constructs (D, E, and F) were collected on glass slides, hybridized with an antisense c-*fms* riboprobe, and counterstained with Wright-Giemsa stain after autoradiography. Lymphoid cells from the same cultures were washed and plated onto NIH 3T3 cells expressing a membrane-bound form of human CSF-1. Plate G shows that early pre-B cells infected with vector alone were unable to form colonies, whereas those expressing wild-type c-*fms* (H) and c-*fms*(969F) (I) formed large colonies by 14 days after plating.

When lymphoid cells expressing wild-type CSF-1R were suspended in agar over NIH 3T3 cell feeder layers producing soluble CSF-1, colonies were obtained, indicating that contact between the lymphoid cells and the artificial stroma was not necessary to stimulate their growth (Table 1). However, cells plated in agar containing saturating concentrations (1 nM) of soluble human recombinant CSF-1 without an underlying feeder layer formed colonies at about a 20-fold lower efficiency, implying that the monolayers produced a collaborating growth-promoting activity. Saturating concentrations of IL-7 (100 U/ml) could support B-cell colony formation in agar in the absence of a feeder layer (Table 1). Moreover, CSF-1R-positive B cells plated in agar containing limiting IL-7 concentrations plus excess soluble human CSF-1 yielded many more colonies than did either factor alone (data not shown), indicating that IL-7 and CSF-1 could act synergistically. However, unlike bone marrow stromal cells, NIH 3T3 cells produce relatively little IL-7, suggesting that it need not represent the collaborating growth factor. The unavailability of neutralizing antibody to IL-7 prohibited a definitive test of this hypothesis.

Although cells infected with the wild-type c-*fms* gene did not rapidly overgrow their uninfected counterparts in medium containing CSF-1, by 17 weeks after establishment, greater than 90% of cells expressed human CSF-1R, as judged by in situ hybridization and fluorescence-activated cell sorting analysis. By this time, cultures expressing CSF-1R(969F) required neither CSF-1 nor stromal cell factors for growth. Moreover, cells infected with c-*fms*(969F) which had never been exposed to CSF-1 also began to rapidly proliferate to high densities and gave rise to FI variants able to grow without stromal support (Fig. 1). Thus, although c-*fms*(969F) does not transform NIH 3T3 cells (49), it can induce factor independence in B cells after prolonged in vitro passage.

Factor-independent B cells expressing CSF-1R(969F),

TABLE 1. Colony formation by human CSF-1R-positive lymphoid cells

Culture	Colony formation on NIH 3T3 monolayers[a] (% of cells plated)				Colony formation in agar[b] (% of cells plated in)		
						CSF-1	
	−CSF-1	+CSF-1(S)	+CSF-1(M)	+CSF-1(M) irradiated	IL-7	Absence of feeder layer	Presence of feeder layer
Uninfected	0	0	0	0	0.8	0	0
c-*fms*	0	0.5	0.6	0.5	0.9	0.3	6.8
c-*fms*(969F)	0.03	0.8	1.5	1.0	1.4	0.7	12.1
v-*fms*	5.4	7.1	10.5	5.1	8.3	4.6	13.4

[a] Lymphoid cultures were infected with c-*fms*, c-*fms*(969F) and v-*fms* retroviruses. Nine weeks later, 10^4 lymphoid cells from the cultures were seeded on monolayers of NIH 3T3 cells (−CSF-1) or on NIH 3T3 cells expressing membrane [+CSF-1(M)] or secreted (+CSF-1(S)] forms of CSF-1 in duplicate 35-mm-diameter dishes containing 2 ml of lymphoid growth medium. One set of CSF-1(M) monolayers was irradiated with 2,500 rads prior to seeding. Colonies were enumerated 2 weeks later.

[b] 10^3 cells from each culture were plated in soft agar supplemented with soluble murine IL-7 (100 U/ml) or soluble human CSF-1 (1 nM) in the presence or absence of an underlying NIH 3T3 feeder layer.

whether passaged in CSF-1 or not, were subcloned at limiting dilution in microdilution wells, and 9 representative FI subclones, each from an independent culture, were selected for further study (Fig. 1). In parallel, factor-dependent cells expressing wild-type CSF-1R were subcloned in microdilution wells containing NIH 3T3 feeder layers expressing membrane-bound CSF-1. Under these conditions, 11 of 12 single-cell-derived clones could not be passaged indefinitely, but we were able to derive one factor-dependent cell line, designated D1F9, that was capable of continuous growth in medium containing either CSF-1 or IL-7 (see below).

Clonality of early pre-B-cell lines expressing CSF-1R. Cellular DNA was extracted from B lymphoid cultures 17 weeks after establishment and digested with *Eco*RI, an enzyme that recognizes a single cleavage site in proviral DNA (Fig. 3A, top). When hybridized with a c-*fms* probe, each integrated provirus should yield a single band whose length would be determined by adventitious sites of *Eco*RI cleavage in host cellular sequences flanking the 5′ end of proviral DNA. Because vector proviruses integrate at random (22), the ability to detect discrete junction fragments rather than a smear of hybridizing bands depends on the frequency at which particular integrations occur within the population.

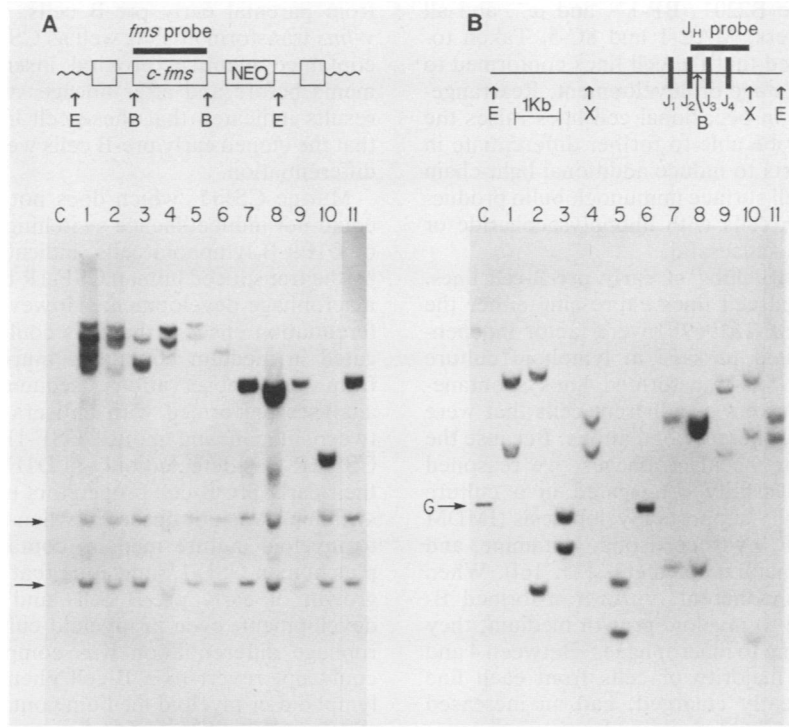

FIG. 3. Proviral integration and immunoglobulin heavy-chain gene rearrangements in infected early pre-B cells. (A) A Southern blot of *Eco*RI-digested DNAs from BALB/c liver (C, control) and from cultures infected with v-*fms* (lanes 1 through 5), c-*fms*(969F) (lanes 6 through 10), and D1F9 cells expressing wild-type c-*fms* (lane 11) was hybridized with a human c-*fms* probe. The top of the panel shows a restriction map of the vector provirus and indicates the probe used. The positions of the major cross-hybridizing endogenous murine c-*fms* fragments are indicated at the left (by arrows). (B) A Southern blot of *Xba*I-digested DNAs from the same cultures shown in panel A (same sample order) was hybridized with a J_H probe. The top of the panel shows a map of the expected 3.6-kilobase J_H-containing *Xba*I fragment present in the mouse germ line (indicated by G to the left of the blot) and designates the probe used for the analysis. Restriction enzymes: E, *Eco*RI; B, *Bam*HI; and X, *Xba*I.

When a radiolabeled human c-*fms* probe was hybridized to normal mouse liver DNA, several background bands were detected, reflecting the presence of cross-hybridizing c-*fms* proto-oncogene sequences (Fig. 3A, control lane C). DNAs from established lymphoid cultures infected with v-*fms* (lanes 1 through 5) or c-*fms*(969F) (lanes 6 through 10) or from c-*fms*-infected D1F9 cells (lane 11) contained additional hybridizing fragments representing discrete sites of proviral insertion. Thus, of the cells originally present in these cultures, a limited number of infected clones had acquired a proliferative advantage by the time of these analyses. All of the v-*fms*-infected cultures contained several independent sites of proviral integration, consistent with the possibility that the cultures were either oligoclonal or that individual clones had acquired several proviral DNA copies. Subcloning of these cells followed by repeated Southern analysis established that the latter was the case (see Fig. 6).

The same cultures were analyzed for the presence of rearranged immunoglobulin heavy-chain genes (Fig. 3B). When the DNAs were digested with *Xba*I and analyzed with a J_H probe, two non-germ line fragments were observed in all but one of the cell lines, indicating that most of the infected cells had undergone J_H rearrangements on both heavy-chain alleles. The one exception was the c-*fms*(969F)-infected cell line shown in lane 6, which appeared to have J_H loci in a germ line configuration. By contrast, by using a J_K probe, a *Hin*dIII fragment indicative of a germ line κ light-chain gene configuration was the predominant band in DNAs from each of the cultures. Only one of the v-*fms*-infected cultures showed definitive evidence of light-chain gene rearrangement (data not shown). The majority of cells in each culture were $B220^+$, BP-1^+, and μ^-, and all lacked the myeloid markers, MAC-1 and 8C-5. Taken together, the results indicated that the cell lines conformed to an early pre-B (or pro-B) stage of development. Rearrangement of light-chain genes in occasional cell lines raises the possibility that these may be able to further differentiate in the B-cell lineage. Attempts to induce additional light-chain gene rearrangements or cell surface immunoglobulin production by treatment of such cells with lipopolysaccharide or IL-4 have so far not been successful.

Hematopoietic lineage switching of early pre-B-cell lines. Although the 25 subcloned cell lines expressing either the v-*fms* gene product or CSF-1R(969F) were factor independent (Fig. 1), on continued passage in lymphoid culture medium, four of eight v-*fms*-transformed lines spontaneously gave rise to rare clusters of adherent cells that were rapidly overgrown by nonadherent populations. Because the adherent cells appeared to be macrophages, we reasoned that they might be successfully propagated in a culture medium which preferentially supports myelopoiesis (IMDM containing 5% FCS, 2-ME, hydrocortisone, glutamine, and extra vitamin and amino acid supplements [15, 16]). When these four subcloned, nonadherent, v-*fms*-transformed B-cell lines were transferred to myeloid growth medium, they underwent lineage switching to macrophages. Between 4 and 14 days of transfer, the majority of cells from each line became adherent and greatly enlarged, had an increased cytoplasm-to nucleus ratio, and contained numerous cytoplasmic vacuoles (Fig. 4D and E). Analysis of each of the four clones showed that the cells stained histochemically for macrophage butyrate esterase activity (Fig. 4E) and were actively phagocytic (Fig. 4F). The cells remained v-*fms* positive but became negative for the lymphoid antigens B220 and BP-1 and instead expressed the macrophage marker, MAC-1 (Fig. 4G). Northern (RNA) blot analysis revealed that sterile (S_μ) transcripts encompassing the μ immunoglobulin constant region were expressed at high levels in the lymphoid cells but were extinguished after lineage conversion (data not shown). After macrophages developed in the cultures, retransfer to lymphoid medium was unable to reverse their phenotype, suggesting that their commitment to the mononuclear phagocyte lineage was irreversible. In contrast, nine FI subclones derived from cultures infected with c-*fms*(969F) did not undergo lineage switching.

Unlike the v-*fms* transformants, the D1F9 cell line expressing wild-type human CSF-1R remained factor dependent and could only be propagated in IL-7 or in conditioned culture medium from NIH 3T3 cells containing human CSF-1. Like that of nonestablished cultures expressing wild-type human CSF-1R (Table 1), the growth of D1F9 cells was potentiated by a soluble growth-promoting activity from NIH 3T3 cells, and the cells grew less well in medium containing purified human recombinant CSF-1 alone. Figure 5 shows that D1F9 cells grew somewhat faster in lymphoid growth medium containing IL-7 versus conditioned medium containing human CSF-1 but could not survive in unsupplemented lymphoid growth medium. When transferred to myeloid growth medium containing human CSF-1, D1F9 cells differentiated to macrophages that were morphologically, antigenically, and functionally indistinguishable from those derived from v-*fms*-transformed B lymphoid cultures (cf., Fig. 4).

Although all cell lines capable of lineage switching to macrophages were derived by endpoint dilution, we reanalyzed their sites of provirus integration and immunoglobulin gene rearrangements to confirm that the macrophages arose from parental early pre-B cells. Figure 6 shows that the v-*fms* transformants, as well as CSF-1R-positive D1F9 cells, contained identical proviral insertions and J_H rearrangements before and after lineage switching. Together, these results indicated that these cell lines were bipotential and that the cloned early pre-B cells were capable of macrophage differentiation.

Murine CSF-1, which does not bind to human CSF-1R, could not induce lineage switching nor support the viability of D1F9 B lymphoid cells, indicating that signals mediated by the transduced human CSF-1R receptor were required for macrophage development. However, once macrophage differentiation ensued, the cells could be continuously propagated in medium containing murine CSF-1. As predicted from these observations, sequential immunoprecipitation analyses performed with antisera able to discriminate between human and mouse CSF-1R confirmed that murine CSF-1R was detected only in D1F9 macrophages but not in their early pre-B-cell progenitors (data not shown). Lineage switching was not observed when the cells were transferred to myeloid culture medium containing IL-7 alone or IL-7 plus human CSF-1, indicating that IL-7 both maintained the growth of early pre-B cells and suppressed macrophage development, even in myeloid culture medium. Once macrophage differentiation was complete, however, the cells could not revert to a B-cell phenotype and died either in lymphoid or myeloid medium containing IL-7 alone, demonstrating that differentiation was accompanied by a loss of IL-7 responsiveness.

Because human CSF-1 was required for D1F9 to carry out a differentiation program characteristic of mononuclear phagocytes, the cells were cloned in agar in lymphoid medium containing IL-7, and single colonies growing in response to IL-7 were picked and expanded. As expected, the colonies had a compact lymphoid morphology, expressed B220 and

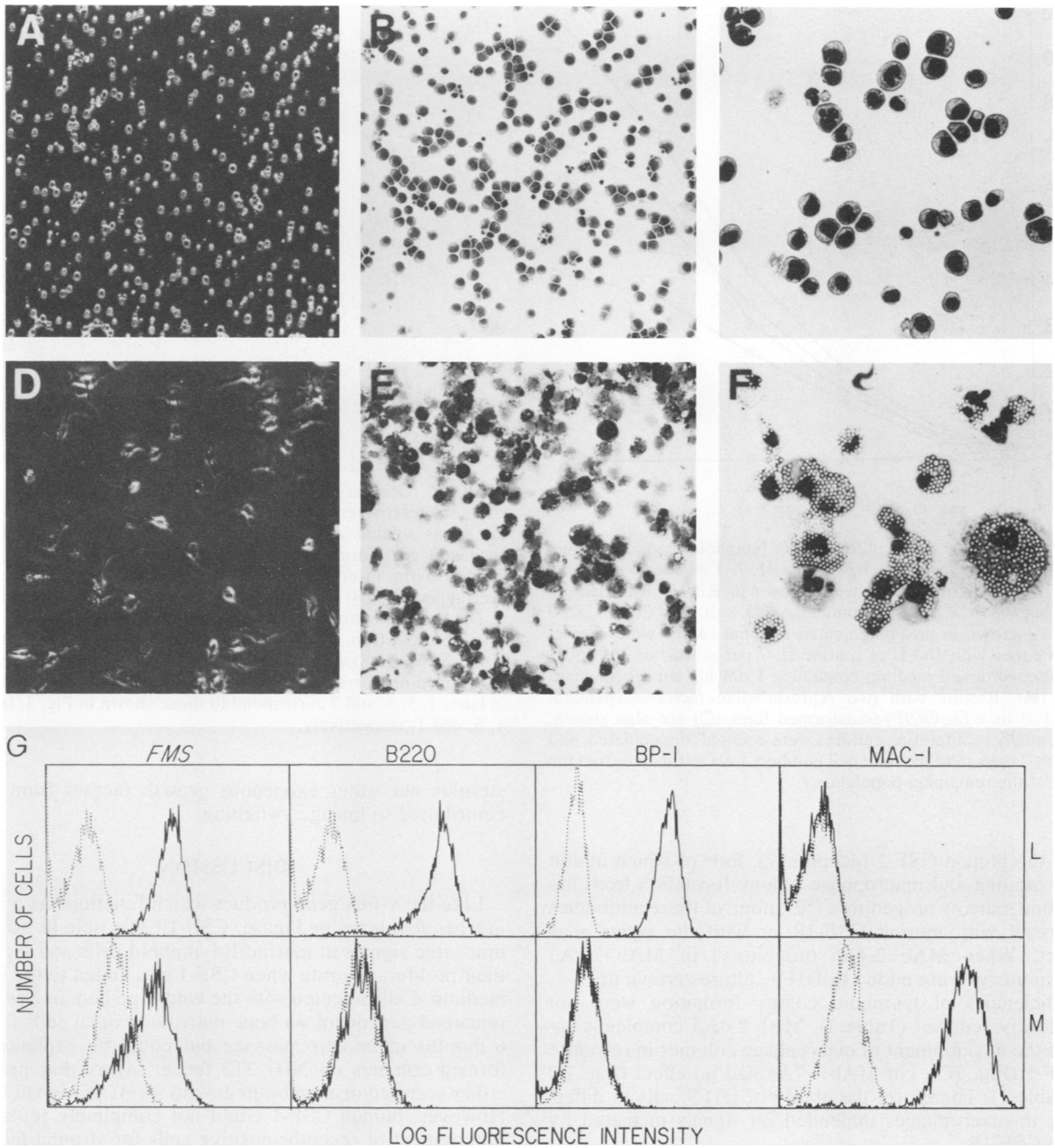

FIG. 4. Differentiation of early pre-B cells to macrophages. Phase-contrast micrographs (A and D), cytocentrifuge preparations stained for butyrate esterase (B and E), and cells incubated with 0.03-μm latex beads (C and F) are shown (matched magnifications) before (A through C) and after (D through F) lineage switching. Panel G shows flow cytometric analysis of cell surface antigens [*fms*, B220 (14.8), BP-1, and MAC-1] on cells with lymphoid (L, top) and macrophage (M, bottom) characteristics. Positively stained cells (solid lines) are compared to background fluorescence (dotted lines) obtained with isotype-matched control antibodies.

BP-1 antigens, and lacked detectable MAC-1 on their surface. The cells were then replated in agar over NIH 3T3 cell feeder layers in myeloid growth medium containing either IL-7 or soluble human recombinant CSF-1. Cells plated in the absence of growth factors over NIH 3T3 feeder layers rarely formed colonies (Table 2). When IL-7 was added to the cultures, their cloning efficiency was greatly enhanced (Table 2) and the colony morphology remained compact (Fig. 7A) and consisted only of lymphoid cells. However, when the cells were seeded in agar containing purified human recombinant CSF-1, diffuse colonies typical of macrophages from normal mouse bone marrow were obtained (Fig. 7B and Table 2). Examination of these cells again revealed that they were macrophage in type, lacked B-cell antigens, were esterase positive and phagocytic, and expressed MAC-1. As in liquid culture, when both IL-7 and CSF-1 were added together, colonies were obtained at an even higher efficiency (Table 2) but macrophage development was inhibited.

We recently developed 12 MAbs to extracellular epitopes

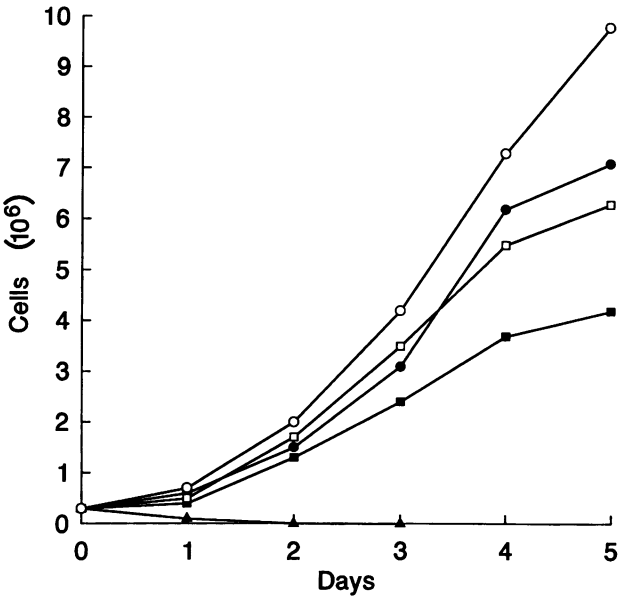

FIG. 5. Rates of cell proliferation in lymphoid medium supplemented with human CSF-1 or murine IL-7. Cells seeded at 3×10^5/ml in T25 culture flasks were grown in RPMI 1640 medium containing 5% FCS, 2 mM glutamine, and 5×10^{-5} M 2-ME. D1F9 cells were grown in unsupplemented medium (▲) or were supplemented either with 100 U of murine IL-7 per ml (●) or with NIH 3T3-cell-conditioned medium containing 1 nM human recombinant CSF-1 (■). Results with two representative factor-independent v-*fms*- (○) or c-*fms*(969F)-transformed lines (□) are also shown. Exponentially proliferating cultures were counted, depopulated, and fed every 2 days, and the total cell numbers were estimated from the growth of the remaining populations.

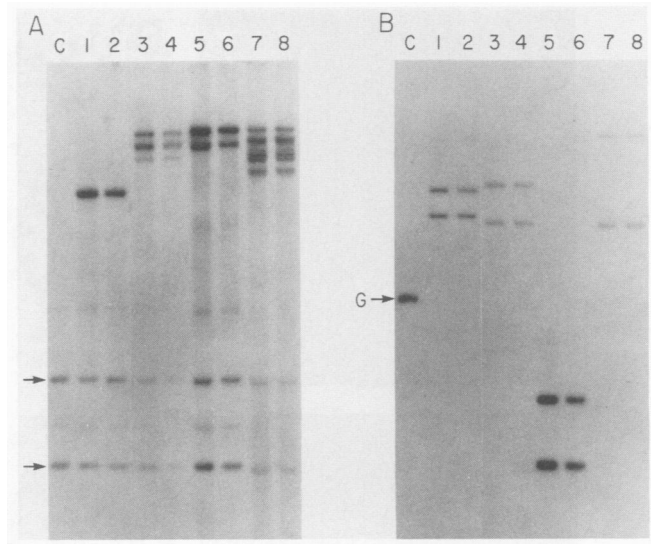

FIG. 6. Comparison of proviral insertion sites and immunoglobulin gene rearrangements in cells before and after lineage switching. DNA was isolated from parental early pre-B cultures. The cells were then induced to form macrophages, and DNA was reisolated 14 days later. Proviral insertion sites (A) and immunoglobulin heavy-chain rearrangements (B) were analyzed as described in the legend to Fig. 3. DNAs from control mouse liver (lane C), from D1F9 cells (lanes 1 and 2), and from three factor-independent v-*fms*-infected clones (lanes 3 through 8) were compared before (odd-numbered lanes) and after (even-numbered lanes) lineage switching. The cell lines shown in lanes 1, 3, 5, and 7 correspond to those shown in Fig. 3, lanes 11, 4, 5, and 1, respectively.

within the human CSF-1 receptor (2), four of which inhibit CSF-1 binding and macrophage colony formation from human bone marrow progenitors (52); none of these antibodies cross-react with murine CSF-1R or with the v-*fms* gene product. When MAb 2-4A5 (inhibitory) or MAb 7-7A3 (noninhibitory) were added to D1F9 cultures grown in IL-7, the efficiencies of lymphoid colony formation were not significantly reduced (Table 2). MAb 2-4A5 completely inhibited the development of macrophage colonies in response to CSF-1 (Fig. 7C), but MAb 7-7A3 had no effect (Fig. 7D and Table 2). Therefore, the ability of D1F9 cells to differentiate to macrophages depended on signals mediated by human CSF-1R.

Analysis of other media requirements. Although the differentiation of early pre-B cells to macrophages required the transduced human c-*fms* gene, their accelerated development in myeloid growth medium indicated that other variables could contribute to lineage switching. Supplementing RPMI 1640 (used for lymphoid medium) with serum, hydrocortisone, or vitamin and amino acid supplements from myeloid medium did not potentiate macrophage development in the presence of CSF-1. In contrast, removal of 2-ME from lymphoid medium induced a greater proportion of cells to become adherent, whereas substitution of IMDM for RPMI 1640 represented the major variable. A comparison of the ingredients in RPMI 1640 and IMDM reveals numerous differences in their concentrations of inorganic salts, amino acids, and other metabolites, so that we have not as yet determined which compounds are critical for alterations in cell phenotype. However, these studies indicate that neither steroids nor other exogenous growth factors from serum contributed to lineage switching.

DISCUSSION

Like the v-*fms* gene product which functions as a ligand-independent tyrosine kinase, CSF-1R was able to transduce mitogenic signals in murine B lymphoid cells and increased their proliferative rate when CSF-1 was added to the culture medium. Cells infected with the wild-type human c-*fms* gene remained dependent on bone marrow stromal cells for over 6 months of in vitro passage but could be explanted and formed colonies on NIH 3T3 feeder layers that produced either secreted or membrane-bound forms of human CSF-1. However, human CSF-1 could not completely replace the requirement of receptor-positive cells for stromal factor(s),

TABLE 2. Inhibition of macrophage colony formation by MAbs to human CSF-1R[a]

Hemopoietin added	Addition of antibody: None	MAb 2-4A5	MAb 7-7A3	Colony phenotype
None	0.2	0	0.1	Compact lymphoid
CSF-1	9.6	0	10.3	Diffuse macrophage
IL-7	15.2	12.4	12.1	Compact lymphoid
CSF-1 plus IL-7	20.6	13.5	22.1	Compact lymphoid

[a] D1F9 cells were plated in soft agar (10^3/ml) with various combinations of CSF-1 (1 nM), IL-7 (100 U/ml), and MAbs to human CSF-1R. The latter include MAb 2-4A5 (inhibitory) and MAb 7-7A3 (noninhibitory). All colony-forming assays were performed in the presence of an NIH 3T3 feeder layer. After 12 days, triplicate plates were counted and colony phenotypes were scored. The numbers indicate the percentage of plated cells that formed colonies.

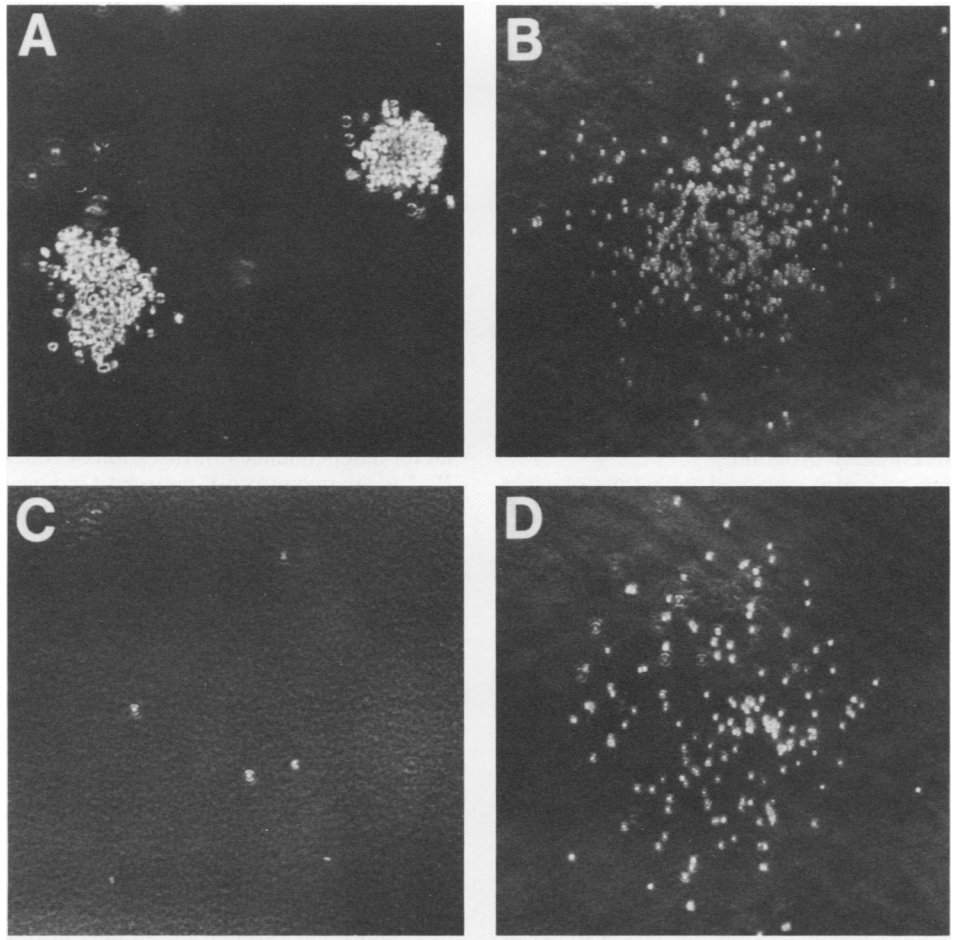

FIG. 7. Phenotype of D1F9 colonies and inhibition of macrophage differentiation by antibodies to human CSF-1R. D1F9 cell colonies plated in semisolid medium supplemented with IL-7 were picked and reseeded as single cells in agar over NIH 3T3 feeder layers in the presence of IL-7 (A) or CSF-1 (B). Individual cells were also seeded in CSF-1 in the presence of 5 μg of purified, serum-free MAb 2-4A5 (C) or MAb 7-7A3 (D) per ml. Both MAbs react to epitopes in the extracellular domain of human CSF-1R, but only MAb 2-4A5 inhibits CSF-1 binding to its receptor (52).

because the pre-B cells could not be maintained in liquid cultures containing soluble human recombinant CSF-1 alone. The stromal cells in long-term lymphoid cultures produce IL-7 (39, 40), which appears to be critical in ensuring early pre-B-cell development. Explanted pre-B cells, whether CSF-1R-positive or not, were unable to form colonies on normal NIH 3T3 monolayers, suggesting that the level of IL-7 production by these feeder layers, if any, was insufficient to support B lymphoid cell growth. In contrast, soluble human CSF-1 plus limiting concentrations of IL-7, soluble CSF-1 plus NIH 3T3 feeders, or simply, NIH 3T3 cells engineered to produce CSF-1 all supported colony formation of human CSF-1R-positive B lymphoid cells. Thus, the requirement for NIH 3T3 cell feeders could reflect synergy between CSF-1 and low concentrations of IL-7 or, alternatively, production of yet another soluble growth-promoting activity by these cells (50a).

After cloning by limited dilution on CSF-1-producing feeder layers, the D1F9 subclone was found to have sustained an immortalizing event which enabled the cells to grow continuously in liquid medium containing CSF-1 or IL-7. Cells with this phenotype were not obtained from 11 other cultures infected with the same vector, although only the culture that gave rise to the D1F9 variant was subjected to limited dilution analysis. When shifted to myeloid growth medium containing human CSF-1 alone, D1F9 cells became adherent and differentiated to macrophages, but high concentrations of IL-7 suppressed their differentiation, even if CSF-1 was present. The cells retained immunoglobulin gene rearrangements characteristic of their lymphoid progenitors but no longer expressed sterile μ transcripts; macrophages expressed MAC-1 antigen in lieu of the pre-B-cell markers B220 and BP-1, became esterase positive, and were actively phagocytic. Most importantly, lineage switching occurred only in myeloid growth medium containing human (but not mouse) CSF-1 and was specifically inhibited by a MAb directed to human CSF-1R. These results demonstrated that signals mediated through the human CSF-1 receptor were necessary to induce lineage switching. As in normal bone marrow cells, expression of the endogenous murine c-*fms* gene increased as macrophage development ensued, and the differentiated macrophages could then be maintained in either murine or human CSF-1. However, once the cells differentiated to macrophages, their retransfer to medium containing IL-7 neither reverted their phenotype nor allowed their survival. Together, these data provide direct evidence that the CSF-1R kinase can not only support cell proliferation and survival but can, in these circumstances, influence

the expression of genes that determine mononuclear phagocyte differentiation.

In addition to a requirement for CSF-1, lineage switching of D1F9 cells occurred only in myeloid growth medium. Although we considered the possibility that hydrocortisone might play a role in inducing macrophage development, D1F9 cells, like v-*fms*-transformed pre-B cells (7), are steroid resistant. Removal of 2-ME, which is normally required for B lymphoid growth in vitro (9), and the use of IMDM were found to contribute independently to macrophage differentiation. In contrast to lineage switching observed with B-cell lines coexpressing the *myc* and v-*raf* oncogenes (29), elimination of 2-ME was in itself insufficient to ensure macrophage development, and the combination of IMDM addition and 2-ME removal ensured a maximal differentiative response when CSF-1 was present. Therefore, other exogenous growth factors, such as those found in FCS, did not induce the differentiative program.

Lineage switching was also obtained with four of eight factor-independent v-*fms*-transformed B-cell lines tested, suggesting that the constitutively active v-*fms* tyrosine kinase can substitute for CSF-1R-mediated signals. The v-*fms* transformants were extensively passaged in vitro and no longer exhibited stromal cell dependence. Unlike D1F9 cells, we saw some evidence of their spontaneous macrophage differentiation in lymphoid medium, even in the presence of IL-7, but the proportion of adherent cells remained low until the cells were transferred to IMDM. D1F9 cells stimulated by IL-7 are precluded from macrophage differentiation, whereas factor-independent v-*fms* transformants appear to stochastically differentiate toward macrophages at a low frequency even under culture conditions that preferentially support lymphopoiesis. Other factor-independent v-*fms* transformants could not differentiate toward macrophages. Moreover, the c-*fms*(969F) gene, which fails to transform fibroblasts but encodes a hyperresponsive form of CSF-1R (49), initially conferred a more robust CSF-1-dependent proliferative response and, unexpectedly, induced the appearance of rapidly proliferating, factor-independent cells after their prolonged passage in medium lacking CSF-1. These cells, like some v-*fms* transformants, appeared to have undergone transforming events which enhanced their proliferative rate, abrogated IL-7 dependence, and mitigated against their lineage conversion.

Commitment to the lymphoid or myeloid lineages appears to occur early during hematopoiesis, and macrophages normally arise from multipotential myeloid precursors that are also capable of differentiating to granulocytes and erythroid cells. However, human leukemias displaying characteristics of pre-B and myeloid lineages have been described and are either thought to result from genetic misprogramming (34) or to arise directly from normal bipotential progenitors (18). Like these neoplastic cells, immortalization of D1F9 cells or v-*fms*-transformed clones may have contributed to their ability to exit from the B-cell developmental pathway and undergo lineage conversion. Murine hematopoietic cells with both pre-B and myeloid characteristics have similarly been generated by in vitro transformation of bone marrow cells with oncogenes of the *ras* and tyrosine-kinase gene families (21). A v-*ras*-transformed B lymphoid line (HAFTL-1) ontologically antedated previously classified early pre-B-cell lines and was induced to mature in the B-cell lineage by bacterial lipopolysaccharide. However, single-cell cloning of the stimulated HAFTL-1 cells gave rise to mixed lymphoid and myeloid clones from which macrophages spontaneously arose (14). Similarly, 5-azacytidine treatment of v-*abl*-transformed ABLS-8.1 pre-B cells yielded macrophages (8). The ability to clone murine c-*fms* cDNA from a pre-B-cell library (47) suggests that low levels of murine CSF-1R expression might also contribute to lineage switching of some such lines.

Pre-B cells derived from mice expressing a *myc* transgene driven by an immunoglobulin enhancer (Eu-*myc* mice) underwent lineage switching to macrophages after infection in vitro with a retrovirus containing the v-*raf* oncogene (29). All of the Eu-*myc*-transformed lines were able to proliferate as B cells without exogenous growth factors, suggesting that their immortalization abrogated their response to IL-7 (or other growth factors) that are normally required for B-cell proliferation. By contrast, murine macrophage tumors induced by a c-*myc*-containing retrovirus were initially CSF-1-dependent (4) but underwent secondary genetic alterations (e.g., production of GM-CSF or CSF-1) leading ultimately to their factor independence (5). Insertion of genes such as v-*raf* into IL-7-independent pre-B cells may induce a subset of master genes that govern macrophage development, and a unifying hypothesis is that the latter genes can be conditionally regulated by CSF-1R. The activity of the c-*raf*-1 serine kinase is enhanced by platelet-derived growth factor (38), and the structural relationship between CSF-1R and B-type platelet-derived growth factor receptor (12, 45, 60) suggest that c-*raf*-1 might also function as an intermediate in the CSF-1R signal transduction pathway. If this were the case, transduction of the B-type platelet-derived growth factor receptor into pre-B cells might mimic effects of CSF-1R. Moreover, in this biologic setting, the constitutive activities of oncogenic *fms*, *raf*, *ras*, and *abl* genes might also exert their differentiative effects by inducing CSF-1-responsive genes. Whether or not similar responses might be mediated by other receptor kinases or oncogene products, our data provide direct evidence that CSF-1R may not merely serve to support cell survival in the face of differentiative signals mediated by other CSFs and interleukins but rather can exert a conditionally deterministic force of its own.

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