Monoclonal Antibodies to the Human CSF-1 Receptor (c-fms Proto-Oncogene Product) Detect Epitopes on Normal Mononuclear Phagocytes and on Human Myeloid Leukemic Blast Cells

By Richard A. Ashmun, A. Thomas Look, W. Mark Roberts, Martine F. Roussel, Stephanie Seremetis, Masahiro Ohtsuka, and Charles J. Sherr

The first monoclonal antibodies (MoAbs) to epitopes in the extracellular domain of the human c-fms proto-oncogene product (receptor for the macrophage colony stimulating factor, CSF-1) were used with flow cytometric techniques to study receptor expression on normal human peripheral blood monocytes, bone marrow cells, and leukemic blasts. On normal cells CSF-1 receptors were restricted in their expression to cells of the mononuclear phagocyte lineage. CSF-1 receptors were detected on leukemic blasts from 15 (30%) of 50 children with acute myeloid leukemia, compared with four (15%) of 26 adults. By contrast, detectable CSF-1 receptors were uniformly absent on blasts from 19 children with acute lymphoblastic leukemia. CSF-1 receptors on normal monocytes and myeloid leukemia cells could be induced to downmodulate by incubation with either human recombinant CSF-1 or phorbol esters, confirming that the receptors had functional ligand-binding sites and responded to transmodulation by inducers of protein kinase C. The numbers of receptors per cell and the percentage of positive cases were highest for leukemic blasts with cytochemical and morphological features of monocytes. However, CSF-1 receptors were also detected on a subset of leukemic blast cells with features of granulocytic differentiation (FAB subtypes M1 through M3). Southern blotting analyses of DNA from 47 cases of acute myeloid leukemia demonstrated no rearrangements within the 32 kb of genomic sequences that contain CSF-1 receptor coding exons or in the 50 kb upstream of the first coding exon. Analysis of the upstream region of the c-fms locus revealed that sequences representing the terminal 112 untranslated nucleotides of c-fms mRNA map 26 kb 5' to the first coding exon, suggesting that at least one c-fms promoter is separated from the receptor coding sequences by a very long intron. Whereas expression of the CSF-1 receptor in myeloid leukemic blasts is not restricted to cells with monocytic characteristics, the apparently aberrant pattern of receptor synthesis in a subset of cases with granulocytic features appears not to be due to chromosomal rearrangements within 50 kb upstream of sequences encoding the receptor.

The c-fms proto-oncogene encodes the receptor for the macrophage colony-stimulating factor, CSF-1 (M-CSF), a hemopoietin whose actions are required for the growth and survival of cells of the mononuclear phagocyte lineage. During normal hematopoiesis, CSF-1 receptors are first expressed on cells committed to the mononuclear phagocyte lineage and, as the cells differentiate, the number of receptors per cell increases markedly. Thus expression of the CSF-1 receptor (CSF-1R) serves as an excellent differentiation-specific marker for mature monocytes and macrophages. Upon binding its ligand, CSF-1R exhibits an intrinsic tyrosine-specific protein kinase activity, resulting in its phosphorylation on tyrosine, accelerated receptor turnover (downmodulation), and the phosphorylation of other intracellular proteins, at least some of which are likely to be essential in transducing mitogenic signals from the plasma membrane to the cell nucleus.

An appealing hypothesis is that critical genetic alterations in the structure of CSF-1R might contribute to an abnormally sustained mitogenic stimulus, resulting in the malignant transformation of hematopoietic target cells. The feline retroviral oncogene, v-fms, encodes an analogue of CSF-1R that is constitutively phosphorylated on tyrosine and exhibits high basal levels of tyrosine-specific protein kinase activity in the absence of its ligand. These functional differences are due to genetic alterations in the v-fms gene that enable it to transform cells by a nonautocrine mechanism. Introduction of the feline v-fms gene into murine CSF-1 or interleukin-3-dependent myeloid cell lines rendered the cells both factor independent and tumorigenic, whereas lethally irradiated mice engrafted with bone marrow cells expressing the v-fms gene developed leukemias and myeloproliferative syndromes involving multiple hematopoietic lineages.

Inappropriate expression of the unaltered CSF-1 or c-fms genes in hematopoietic cells might also contribute to myeloproliferative disorders or to overt malignancy. Cotransduction of the human CSF-1 and c-fms genes into mouse NIH-3T3 cells resulted in transformation and tumorigenicity by an autocrine mechanism. Similarly, introduction of the human CSF-1 gene into SV-40 immortalized CSF-1-dependent mouse macrophages rendered them independent of the exogenous growth factor for proliferation and survival.

From the Departments of Tumor Cell Biology and Hematology-Oncology, St. Jude Children's Research Hospital and the Howard Hughes Medical Institute, Memphis; the Department of Rheumatology, Hospital for Joint Diseases, New York; and the Division of Hematology-Oncology, Department of Pediatrics, the University of Tennessee, Memphis, College of Medicine.

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Address reprint requests to Dr Charles J. Sherr, Department of Tumor Cell Biology, St. Jude Children's Research Hospital, PO Box 318, Memphis, TN 38101.

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sequences in the In contrast, retroviral integration conventional Southern blotting procedures to assay for rear-
tumor formation. contribute to that with acute of patients after treatment with genic in nude mice. Normal human monocytes can be survival in culture, although the cells remained nontumori-
genic in nude mice. Normal human monocytes can be transiently induced to produce CSF-1 after treatment with γ-interferon and granulocyte-macrophage–colony-stimulat-
ing factor (GM-CSF); however, blast cells from a subset of patients with acute myelogenous leukemia (AML) were found to persistently express both CSF-1 and its receptor, suggesting that an autocrine mechanism might underlie their abnormal proliferation. In contrast, retroviral integration upstream of CSF-1R coding sequences in mice results in aberrant receptor expression in immature myeloid cells, leading to their proliferative expansion and eventual leukemic conversion. Presumably aberrant expression of high levels of CSF-1R in immature myeloid progenitor cells renders them inappropriately responsive to the growth fac-
tor. In some cases factor-independent leukemic clones arising either in vivo or in ex vivo culture were found to produce CSF-1, whereas other leukemic clones were found to be homozygous for the rearranged c-fms allele, suggesting that they may have undergone subsequent genetic alterations in CSF-1R coding sequences. Thus unregulated expression of either CSF-1 or its receptor in myeloid progenitors can contribute to tumor formation.

The CSF-1 and CSF-1R (c-fms) genes have been assigned to the long arm of human chromosome 5, together with a group of genes encoding other hemopoietins, another growth factor, growth factor receptors, a myeloid differ-
entiation antigen, and a putative transcriptional regulatory factor. Acquired interstitial deletions of chromosome 5q are a frequent abnormality in leukemic blasts of adults who develop AML after exposure to toxic chemicals, and deletions within this region are also characteristic of the “5q– syndrome,” a refractory anemia presenting with thrombocy-
tosis and abnormal megakaryocytes. These findings raise the possibility that genetic rearrangements affecting one or more of these genes might also predispose to myelodysplastic or leukemic processes.

With these collective observations in mind, we molecularly cloned sequences 5' to the human c-fms gene and used conventional Southern blotting procedures to assay for rearrangements either upstream or within c-fms coding sequences in the DNA of leukemic blasts from patients with AML. In parallel, we measured the levels of expression of CSF-1R on leukemic blasts to determine if we could discern patterns of receptor expression that could be related to malignant transformation. For the latter studies, polyclonal antisera and monoclonal antibodies (MoAbs) specific for epitopes in the extracellular ligand binding domain of the CSF-1 receptor were developed and used for flow cytometric measurement of receptors on the surface of both normal and leukemic cells. Although rearrangements of the c-fms gene and its proximal 5' flanking sequences were not detected in an extensive survey of patient samples, we documented expression of CSF-1 receptors on blasts from a high percent-
age of myeloid leukemias of both children and adults, including some cases that lacked morphological and cyto-
chemical characteristics associated with monocytic differen-
tiation.

MATERIALS AND METHODS

Cells and culture conditions. Normal human peripheral blood was collected in heparin and immediately placed on ice. Cells were separated by centrifugation at 4°C after layering over cold Ficoll-
Hypaque (FH) solution (specific gravity, 1.357) (Pharmacia Fine Chemicals, Piscataway, NJ). The buoyant mononuclear cells were washed in cold Hanks' balanced salt solution (HBSS), resuspended in cold RPMI 1640/10% fetal calf serum (FCS), 1-glutamine, antibiotics, and 10 mmol/L HEPES, and then analyzed immediately by flow cytometry or placed in culture overnight at 37°C for analysis the following day. Mononuclear cells to be analyzed on the day of collection were washed and resuspended in cold staining medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 5% FCS, 10 mmol/L HEPES, 1-glutamine, antibiotics, and 2 mmol/L sodium azide). Granulocytes were isolated from the pellet of the FH separation after lysis of RBCs, as previously described; all procedures were performed at 4°C in the presence of 2 mmol/L sodium azide. In some experiments, mononuclear cells were divided into three aliquots, cultured overnight, and either left untreated or treated for 90 minutes with medium containing saturating amounts of recombinant human CSF-1 (10,000 U/mL) or with 10–4 mol/L 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St. Louis). The cells were harvested by scraping after addition of three volumes of ice-cold staining medium to the tissue culture flasks.

Children whose leukemic cells were studied were admitted to St. Jude Children's Research Hospital, Memphis; adults were admitted to Mount Sinai Medical Center, New York. All patients were advised of procedures and risks in accordance with institutional guidelines and gave informed consent. Bone marrow cells from patients with acute lymphocytic leukemia (ALL) in remission who had completed chemotherapy for more than 2 years were collected into preservative-free heparin at the time of scheduled bone marrow aspiration. Cells were immediately placed on ice and separated over FH solution at 4°C in the presence of 2 mmol/L sodium azide. Buoyant mononuclear cells were resuspended in cold staining medium and stained for flow cytometric analysis and sorting. Leukemic blasts collected at diagnosis from peripheral blood or bone marrow of pediatric or adult patients with AML were viably frozen in medium containing 10% dimethylsulfoxide (DMSO) after fractionation on FH. Leukemic cell morphology was classified according to the French-American-British (FAB) criteria, based on characteristics of Wright's-stained preparations and reactivity with chloroa
cetate esterase, peroxidase, and α-naphthyl butyrate esterase. Cryo-
preserved leukemic blasts were thawed into RPMI 1640/20% FCS on ice, washed twice in medium at 4°C, and then cultured overnight in RPMI 1640/10% FCS at 37°C. On the following morning, cells were harvested as described above. For a subset of patients, triplicate cultures were either left untreated, treated with CSF-1, or treated with TPA as described for normal monocytes.

Expression of CSF-1 receptors on the surface of normal or leukemic cells was labile and sensitive to even brief periods of exposure to temperatures above 4°C. Fresh cell samples from blood or bone marrow had to be chilled immediately and kept cold during subsequent staining and analysis. Under these conditions, CSF-1 receptors were readily detected on either fresh or cultured samples. However, because some leukemic samples were originally collected and separated at room temperature before cryopreservation, CSF-1R might have been downmodulated; thus frozen leukemic cells were analyzed only after overnight culture, thereby permitting the cells to upregulate receptors. Although most leukemic blasts were nonadherent to plastic in overnight culture, receptor synthesis may have been induced through cell attachment. Moreover, the levels of receptors expressed by cultured cells may not reflect the actual

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receptor number on circulating cells in vivo where CSF-1 normally is present as a component of normal serum.

Production of antibodies to human CSF-1-R. MoAbs and polyclonal mouse antisera that bind specifically to epitopes in the extracellular domain of human CSF-1R were produced by procedures similar to those used to prepare antibodies to the v-fms gene product. Tumorigenic rat NRK cells transformed by the Kirsten strain of murine sarcoma virus were infected with a helper-free retrovirus containing both the human c-fms gene and the gene for neomycin resistance. Retroviral stocks were produced in the amphotropic packaging cell line PA317\textsuperscript{**} after selection of transfected cells with G418 (Sigma Chemicals, St. Louis). A G418-resistant single-cell clone was selected that expressed high levels of human CSF-1R, as determined by an in vitro assay for c-fms-coding kinase activity.\textsuperscript{46} Receptor-positive cells were injected subcutaneously into nine-day-old Lewis or Sprague-Dawley rats (5 × 10\textsuperscript{6} cells per animal). Animals developed solid tumors and mounted an immune response to the human c-fms gene product within 3 weeks. Spleens from animals producing high titers of c-fms-specific antiserum were fused to rat Y3-Agl.2.3 myeloma cells,\textsuperscript{46} and hybridoma supernatants derived from five independent fusions were screened by flow cytometric procedures using NIH-3T3 cells expressing a transduced human c-fms gene (see below). Cells from antibody-positive wells were cloned once at limiting dilution and restated by flow cytometry; positive cultures were recloned from single colonies in semisolid medium and established as stable cell lines. Twelve different rat hybridomas produced immunoglobulin (Ig) G, and two additional lines produced IgM. All cell lines could be propagated in serum-free medium,\textsuperscript{46} thereby enabling purification of Igs to homogeneity by conventional methods. Five such antibodies were used for this study. Isotype-matched rat MoAbs reactive to the v-fms gene product but nonreactive to c-fms-coding epitopes\textsuperscript{46} were used as controls for flow cytometric analyses.

Mouse antisera specific for c-fms epitopes was produced by an analogous procedure. Tumorigenic NIH-3T3 mouse fibroblasts transformed by the Harvey ras oncogene were cotransfected with a c-fms retrovirus together with the plasmid, pSV2neo.\textsuperscript{37} A single-cell clone selected with G418 that expressed high levels of the human c-fms gene product was grown up and injected subcutaneously into nine-day-old NFS mice. The mice developed tumors and mounted an immune response within 3 weeks. Polyclonal antisera from multiple responding animals were pooled and used for flow cytometric analysis\textsuperscript{47}; nonimmune mouse serum was used as a control.

Immunofluorescence staining and flow cytometry. Cells were labeled for flow cytometric study by indirect immunofluorescence as previously described.\textsuperscript{35} Cells at 4°C were incubated for 30 minutes on ice in cold staining medium containing 0.1% BSA and 1% rat IgG (Sigma). After two washes in cold staining medium, the cells were incubated for 30 minutes at 4°C with a titered excess of c-fms-specific antisera, MoAb, or matched control reagents. After two washes in cold staining medium, the cells were incubated for 30 minutes on ice with a fluoresceinconjugated affinity-purified goat antiserum to mouse or rat IgG (Tago, Burlingame, CA). The cells were washed twice more and resuspended in cold staining medium containing 0.25 mM propidium iodide. Stained cells were analyzed with a Coulter EPICS 753 flow cytometer (Coulter Corp, Hialeah, FL). Two-dimensional analysis of forward-angle v orthogonal light scatter was used to identify separate subpopulations of cells within each sample. Fluorescein fluorescence was detected for each subpopulation using two-dimensional, bit-mapped gating of light scatter data. Dead cells labeled with propidium iodide were excluded from the analysis.\textsuperscript{35} A sample of normal human mononuclear cells was analyzed to establish the light-scattering properties of normal lymphocytes and monocytes.

Fluorescent blasts from each patient were analyzed with one or more bit-mapped light scatter gates distinct from those of normal monocytes. Fluorescein fluorescence was measured separately for each subpopulation of cells.

Flow cytometric studies were performed with c-fms-specific polyclonal mouse antiserum and, for a subset of cases, with the panel of five rat MoAbs directed against c-fms-coded epitopes. A leukemic sample was determined to be positive for c-fms expression if greater than 20% of blast cells displayed specific binding of c-fms-specific antiserum or MoAb when compared with control histograms. Bone marrow and peripheral blood cells that bound the human CSF-1-R-specific MoAb 3-44A-E4 were sorted by flow cytometry. Cells with background levels of fluorescence (CSF-1–receptor-negative cell population) and those with fluorescence levels exceeding the control (CSF-1–receptor-positive population) were sorted into separate tubes, centrifuged onto slides, stained with Wright's solution, and histochemically evaluated for chloroacetate esterase and alpha-naphthyl butyrate esterase activity.

Cloning of upstream c-fms genomic sequences. The human c-fms-coding region was previously isolated in overlapping cosmid and bacteriophage recombinants, and was shown to span 32 kb of genomic DNA.\textsuperscript{44,56} Both the 4 kb c-fms cDNA\textsuperscript{59} and genomic clones containing c-fms coding exons (A. Hampe, personal communication) have been completely sequenced. The cDNA sequence contains 120 untranslated nucleotides at its 5' end that are not included in the 32 kb of previously characterized c-fms genomic sequences.\textsuperscript{56} To obtain genomic clones that included the most 5' untranslated sequences of the c-fms cDNA, two overlapping cosmid clones (cosmids A and B, Fig 1) were isolated from a library constructed from human CML DNA provided by Dr W. J. M. Van de Ven.\textsuperscript{57} Cosmid A was isolated by screening 100,000 recombinants using a c-fms cDNA probe and includes genomic sequences 16 kb 5' to the first CSF-1-R coding exon (designated site X in Fig 1). A 0.7 kb BamHI/PstI restriction fragment from the 5' end of cosmid A that lacked human repeated sequences was used as a probe to screen 200,000 additional recombinants; cosmid B contains an additional 44 kb of 5' sequences flanking the receptor locus. A 0.4 kb PstI restriction fragment (designated site Y, Fig 1) from cosmid B,
located 26 kb upstream of the first coding exon of c-fms, hybridized to an oligonucleotide probe complementary to the most 5' untranslated sequences of the c-fms cDNA. This PstI fragment, subcloned into a M13 vector, was sequenced by the dideoxynucleotide chain termination method, and was found to contain the remaining 5' untranslated region of c-fms cDNA. Genomic fragments from the c-fms locus which did not contain repeated human sequences (probes 1-5, Fig 1) were isolated for use as unique sequence probes for Southern blot analysis. The 32 kb CSF-1R coding region was probed using previously derived plasmids (pSM3 and pSM7C) containing regions of the v-fms oncogene.  

Southern blot analysis. DNA was extracted from FH-fractionated leukemic blasts obtained from bone marrow aspirates from 47 patients with AML, including 44 children and three adults. DNAs extracted from human cell lines HPB-ALL, ML-3, or lymphoblastoid cell lines were used as controls. The DNAs were digested with the restriction enzymes EcoRI or XbaI, subjected to electrophoresis in 0.8% agarose gels and transferred to nylon membranes by the method of Southern. The immobilized DNA was sequentially hybridized to seven radiolabeled probes (see above) which, with the enzymes chosen, detected the complete 32 kb c-fms coding sequences as well as 50 kb of 5' flanking sequences. Hybridization and washing of blots were performed using high stringency conditions.  

Other analytic procedures. Metabolic labeling with [35S]methionine, preparation of cell lysates, immunoprecipitation, immune complex kinase assays, and electrophoresis of proteins in gels containing sodium dodecyl sulfate were performed as previously described.  

RESULTS  
Characterization of MoAbs to the human CSF-1 receptor. CSF-1R is an integral transmembrane glycoprotein oriented with its aminoterminal ligand-binding domain on the external surface of the plasma membrane and its carboxyterminal kinase domain in the cytoplasm. It was therefore expected that antibodies raised against live cells expressing the c-fms gene product would be exclusively directed to epitopes in the CSF-1R extracellular domain and would therefore prove useful for flow cytometric measurements of receptors on viable cells. Spleen cells from newborn rats immunized with v-ras-transformed cells expressing a transduced human c-fms gene were fused to rat myeloma cells, and culture supernatants from individual wells were screened by flow cytometry for reactivity to CSF-1R epitopes expressed on mouse NIH-3T3 cells expressing a transfected human c-fms gene. Cells from antibody-positive wells were subcloned, established as stable lines, and grown using serum-free culture conditions to facilitate purification of monoclonal IgGs.  

Using flow cytometric procedures, all of the MoAbs detected epitopes on rat NRK and mouse NIH-3T3 cells transfected with human c-fms-containing vectors but generated background fluorescence profiles with the nontransformed parental cell lines (data not shown). When mouse NIH-3T3 cells expressing human CSF-1R were metabolically labeled with [35S]methionine, lysed with detergent, and the cell lysates immunoprecipitated with different MoAbs, each of the antibodies specifically precipitated molecules with the characteristic mol wt of both the immature intracellular form (gp150\textsuperscript{c-fms}) and mature cell surface form (gp150\textsuperscript{m-m}) of the c-fms-coded glycoprotein (Fig 2A). Because the CSF-1 receptor exhibits an intrinsic tyrosine-specific protein kinase activity, immune complexes containing CSF-1R catalyze autophosphorylation of the receptor on tyrosine residues in reactions performed in vitro in the presence of ATP and manganese ions. As shown in Fig 2B, the mature form of CSF-1R was phosphorylated in immune complexes prepared with different MoAbs, and phosphoamino acid analysis of the labeled receptors confirmed that phosphorylation occurred only on tyrosine residues (data not shown).  

MoAbs reactive with the human c-fms gene product did not detect murine CSF-1R expressed on the surface of an SV40-immortalized mouse macrophage cell line and did not immunoprecipitate the murine c-fms gene product; nor did the antibodies react with v-fms-coded glycoproteins...
MONOCLONAL ANTIBODIES TO HUMAN CSF-1 RECEPTOR

domain might potentially interfere with CSF-i binding to its CSF-iR. We conclude that the MoAbs raised against the human c-fms gene product specifically react with human CSF-1R and do not crossreact with related c- or v-fms-coded antigens expressed by cells of several other mammalian species.

Because MoAbs directed to the CSF-1R ligand-binding domain might potentially interfere with CSF-1 binding to its receptor, we tested several of the MoAbs for their ability to inhibit CSF-1-dependent colony formation in semisolid medium. NIH-3T3 cells expressing the human c-fms gene product were seeded as single cells in agar in the presence of recombinant human CSF-1 and colonies were enumerated 2 weeks later. Preliminary results suggest that four of the antibodies (12-2D6-2C7, 12-3A3-1B10, 12-3A1-2B8, and 2-4A5-8) inhibit the proliferation of these cells in the presence of recombinant human CSF-1 (data not shown). We are currently attempting to determine whether these reagents will also inhibit the CSF-1-dependent formation of macrophage colonies derived from normal human bone marrow progenitors or mediate the complement-dependent lysis of monocytes, macrophages, and their immature precursors.

CSF-1R expression by normal human blood cells. MoAbs directed to epitopes in the extracellular domain of human CSF-1R bound to normal monocytes, but not to lymphocytes or granulocytes (See Fig 3 for representative data with MoAb 3-4A4-E4). A mixed population of WBCs containing monocytes, lymphocytes, and granulocytes was analyzed for forward-angle v orthogonal light-scattering properties and subdivided by electronic bit-mapped gating for fluorescence analysis (see bit-mapped gates in Fig 3D). Only cells having the light-scatter properties of monocytes bound the antibody (Fig 3A), whereas those corresponding to lymphocytes (Fig 3B) or granulocytes (Fig 3C) did not. Buoyant cells purified by FH gradient centrifugation consisted primarily of lymphocytes and monocytes based on light-scatter analysis and were physically separated from granulocytes, which sedimented as a purified population in the FH pellet. Purified granulocytes did not bind MoAbs directed to human CSF-1R. The mixed mononuclear cell fraction (monocytes and lymphocytes) were further fractionated by fluorescence-activated cell sorting (FACS) to morphologically confirm the nature of cells expressing CSF-1R. Sorted receptor-positive peripheral blood mononuclear cells were highly enriched for monocytes, based on their cytometry on Wright's-stained slides and positivity for α-naphthyl-butyrate esterase (Table 1). Conversely, cells failing to bind CSF-1R-specific MoAbs were enriched for lymphocytes, confirming observations based on light-scatter gating that lymphocytes lack CSF-1R.

Similar analyses were performed using normal human bone marrow (Fig 4). Again, mixed populations were electronically subdivided based on their light-scatter properties (Fig 4D) and independently analyzed for fluorescence. Of three major populations with light-scatter properties corresponding predominantly to monocytes (Fig 4A), lymphocytes (Fig 4B), and granulocytic precursors (Fig 4C), only the monocyte fraction exhibited detectable levels of CSF-1R epitopes. These methods are not sufficiently sensitive to preclude that CSF-1R might be expressed by minor populations of mononuclear phagocyte precursors, which were not resolved based on their light scattering properties alone.

Table 1. Characterization of Cells Expressing the CSF-1 Receptor

<table>
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<th>Cell Population</th>
<th>Morphological Classification</th>
<th>Enzymatic Stain</th>
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<td></td>
<td>Mon</td>
<td>Lym</td>
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Abbreviations: CAE, chloroacetate esterase; α-NBE, α-naphthyl butyrate esterase; Mon, monocytes; Lym, lymphocytes; Seg, polymorphonuclear leukocytes; Ban, bands; Mph Pre, myeloid precursors; RBC Pre, erythroid precursors.

*All values are percentages of the total cell population.
†Determined with Wright's-stained preparations.
Flow cytometric studies of CSF-1 receptor expression on leukemic blasts. Using FH fractionated bone marrow mononuclear cells and bit-mapped gating to analyze leukemic cells (Fig 5), CSF-1 receptors were detected on the surface of leukemic blasts from 15 (30%) of 50 children and from four (15%) of 26 adults with AML (Fig 6). Conversely, blast cells from 19 patients with lymphoid leukemia did not express detectable levels of the receptor. Table 2 summarizes results obtained when blast cells from all CSF-1R–positive patients were tested with the CSF-1R–specific polyvalent mouse antiserum, and when those from a subset of patients were tested with the panel of c-fms–specific MoAbs that displayed concordant binding patterns. Two antibodies (12-2D6-2C7 and 12-3A3-1B10) consistently yielded higher fluorescence signal levels, suggesting that they might be especially useful in detecting low numbers of receptors.
Table 2. Flow Cytometric Detection of the CSF-1 Receptor on Human Leukemic Blasts*

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<tr>
<th>Patient No.</th>
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<th>Percent CSF-1R Positive</th>
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Abbreviations: FAB, morphological classification by the French-American-British system; sera, polyvalent mouse antisera; A1, 2-4A5-B (IgG1); A2, 7-7A3-14 (IgG1); A3, 9-4D2-1E4 (IgG1); A4, 12-2D6-2C7 (IgG1); A5, 12-3A3-1810 (IgG1); ND, not done.

*Only patients with detectable levels of receptors are listed.

†Percentage of original patient sample that was leukemic blasts as determined by morphological analysis of Wright’s-stained samples.

‡Percentage of leukemic blasts that expressed detectable levels of CSF-1R as determined by flow cytometric assays.

§Binding is expressed in arbitrary units of fluorescence intensity compared to isotype-matched control antibodies or control sera, where 0 indicates background fluorescence level. Each unit corresponds approximately to a doubling of mean fluorescence intensity.

Receptor expression was demonstrated by leukemic myeloblasts from 62% of children whose blast cells had morphological and cytochemical characteristics of monocytes (FAB classes M4 or M5) and from five other patients whose blasts displayed exclusively granulocytic (FAB classes M2 and M3) or undifferentiated (FAB M1) cytomorphology (ie, 16% of M1 through M3 cases; Fig 6A). Although a lower percentage of adult patients expressed detectable levels of receptors on the surfaces of their leukemic blasts, the receptor-positive cases included three with exclusively granulocytic morphology (M2) (Fig 6B). FACS purification of blasts from selected receptor-positive AML patients confirmed that CSF-1R was expressed by cells that had the morphological and cytochemical features of granulocytic precursors, even though normal granulocytic precursors did not express detectable levels of receptors. The limited availability of cells precluded FACS purification for most patients. Receptor levels were generally highest on the surface of blast cells from patients with monocytic characteristics, as compared to blast cells from patients with granulocytic or undifferentiated cytomorphology.

Fig 7A compares the pattern of CSF-1 receptor expression by blasts from a subset of 29 pediatric cases with that of the CD14 cell surface marker, a 55,000 dalton glycoprotein that is normally found on mature monocytes and macrophages and is recognized by the MoAb MY4. Expression of these two glycoproteins was not correlated, in contrast to findings with mature monocytes. Indeed, seven of 17 pediatric cases negative for MY4 binding expressed CSF-1R. All four adult patients with blasts that expressed CSF-1R were negative for MY4 binding (Fig 7B).

Fig 7. Comparison of CSF-1 receptor and CD14 antigen expression on the surface of leukemic blast cells. Solid bars represent CSF-1R–positive patients; hatched bars represent CSF-1R–negative patients. Patients were classified as CD14-positive if greater than 20% of their blasts expressed the antigen detected by MoAb MY4.
Functional characterization of receptors on leukemic blasts. After binding its ligand, CSF-1 receptor-ligand complexes are internalized in coated pits and rapidly degraded in lysosomes (receptor downmodulation).\textsuperscript{6,10,12,21} Therefore cells bearing functional ligand-binding sites should no longer express receptor epitopes after exposure to saturating concentrations of ligand. To determine whether CSF-1 receptors on leukemic myeloblasts would bind CSF-1 and undergo normal downmodulation, CSF-1R–positive cells were cultured for 90 minutes in the presence of 10,000 U/mL of the human recombinant growth factor and reanalyzed for the presence of detectable receptor epitopes at their cell surface. CSF-1 receptors on leukemic myeloblasts (Figs 8A and 8C) and on normal peripheral blood monocytes (Figs 8B and 8D) were appropriately downmodulated after incubation in human CSF-1–containing medium, indicating that the receptors expressed functional CSF-1R binding sites. All 19 cases of CSF-1R–positive AML expressed functional receptors by this criterion. Moreover, when CSF-1 receptors on leukemic blasts from representative adult AML patients were immunoprecipitated and assayed for immune complex kinase activity, they were autophosphorylated on tyrosine residues and exhibited the apparent electrophoretic mobilities of the immature (gp130\textsuperscript{v-fms}) and mature (gp150\textsuperscript{v-fms}) forms of CSF-1R immunoprecipitated from normal peripheral blood monocytes.

Downmodulation of CSF-1R on leukemic myeloblasts could also be provoked by a 90-minute incubation in medium containing 10\textsuperscript{-6} mol/L TPA (data not shown), similar to the response observed for receptors on normal monocytes incubated with this phorbol ester.\textsuperscript{10,12,60} The “transmodulation” of CSF-1R by inducers of protein kinase C occurs by a mechanism that differs from that of ligand-induced downmodulation.\textsuperscript{12} These results also differ from those obtained with the product of the constitutively activated v-fms oncogene, which undergoes little or no downmodulation after addition of TPA.\textsuperscript{10,12} Thus by these criteria as well, CSF-1R molecules expressed on leukemic cells appeared to be functionally intact.

Southern blot analysis of DNA from leukemic cells. DNAs from leukemic cells of patients with AML were analyzed by Southern blotting to search for rearrangements or deletions within the c-fms proto-oncogene that might account for receptor expression in cells lacking monocytic features. The coding region was analyzed using four distinct probes that hybridized to restriction fragments spanning 21 exons included in approximately 32 kb of DNA (Fig 1). DNAs from leukemic cells of 32 patients were analyzed, including samples from four children (FAB M1 and M2), whose blasts expressed CSF-1R as well as nine children and three adults whose blasts were negative for c-fms expression. No rearrangements of c-fms coding sequences were detected in any of these cases.

Contiguous DNA sequences 60 kb 5′ to the first CSF-1R coding exon were cloned from a cosmid library, and sequences corresponding to 112 of 120 nucleotides from the extreme 5′ end of c-fms mRNA were identified 26 kb upstream (site Y, Fig 1) by hybridization and nucleotide sequencing analysis. These results suggest that additional 5′ exon(s) of c-fms are located at least 26 kb from sequences encoding CSF-1R and imply that the c-fms promoter may reside at an even greater distance from the 32 kb CSF-1R coding block. To survey for upstream gene rearrangements that could potentially affect c-fms transcription, probes hybridizing to restriction fragments spanning a region 50 kb upstream of the first coding exon (Fig 1, probes 1 through 3) were also used to analyze DNA from leukemic blasts of 44 children. These cases included 13 whose blasts expressed c-fms (all five pediatric cases of FAB M1 through M3, Table 2) as well as others whose blasts were receptor-negative. Again, there was no evidence of bands with aberrant mobility that would have suggested rearrangements or deletions in this region of the gene (see Addendum).

DISCUSSION

Using recently prepared MoAbs to the human CSF-1 receptor, we studied leukemic blast cells from 76 patients with acute myeloid leukemia and 19 children with acute lymphoid leukemia for their patterns of CSF-1R expression, using a flow cytometric assay that allowed us to discriminate between receptor expression by malignant blast cells and by cells with the light-scatter properties of normal monocytes. CSF-1R was present on blasts from 30% of cases of childhood and 15% of adult AML cases, but on none of 19 cases of acute lymphoid leukemia tested. The CSF-1R coding sequences were unarranged in all cases studied, and CSF-1R molecules expressed on leukemic cells appeared functionally intact by several criteria. The receptors underwent
ligand-induced and phorbol-ester-induced downmodulation and were active in immune complex kinase assays, indicating that they contained CSF-1R binding sites, were active as tyrosine kinases, and could be appropriately transmodulated by inducers of protein kinase C.

Among normal cells CSF-1R expression was restricted to peripheral blood monocytes and to a subpopulation of bone marrow cells with monocytic features. In contrast, receptor-positive cases of AML included not only those with morphological and cytochemical evidence of monocytic differentiation but also a subset of cases with undifferentiated or granulocytic morphology. The present assays were not sufficiently sensitive to exclude that a minor population of multipotential bone marrow progenitors express CSF-1R or that more mature cells committed to other hematopoietic lineages express the receptor at significantly lower levels. Possibly such cells represent potential targets for malignant transformation and subsequently exhibit lineage infidelity when proliferating as malignant clones. Alternatively, our observations are consistent with the possibility that leukemic myeloblasts from some patients inadvertently express CSF-1R. Unscheduled appearance of CSF-1R might endow otherwise refractory cells with the ability to respond to the exogenous growth factor, thereby directly contributing to their abnormal proliferative expansion and eventual leukemic conversion. Additional genetic alterations relevant to disease progression might then include the activation of CSF-1 synthesis by receptor-bearing cells, as has already been observed in a subset of such cases,27-29 or mutations in the c-fms gene, analogous to those in v-fms,18 which activate the receptor kinase in the absence of its ligand.

The mechanism leading to CSF-1 receptor expression in the subset of human leukemias that lack other characteristics of monocytic differentiation remains unknown. In mice, proviral insertion upstream of the first coding exon of the murine c-fms gene has been clearly implicated as an etiologic factor in 30% of Friend virus-induced myeloblastic leukemias and leads to the inappropriate expression of CSF-1R at high levels in immature myeloid progenitor cells.30 Although the affected cell population may initially be CSF-1 dependent, additional genetic rearrangements upstream of the c-fms coding region in human AML cells could affect the c-fms promoter and induce CSF-1R mRNA transcription. However, we were unable to detect genetic rearrangements in sequences 50 kb 5' to the CSF-1R coding region in any of 44 pediatric cases examined, including five cases classified as FAB M1 through M3.

Our data suggest that the c-fms promoter may be located more than 26 kb upstream of the 21 CSF-1R coding exons, which are themselves distributed over 32 kb. This conclusion is based on the molecular localization of sequences complementary to the extreme 5' untranslated region of a "full-length" c-fms cDNA clone55 encoding a biologically active receptor.18 However, we cannot exclude that there are alternative sites for transcription initiation within the c-fms locus. Oligonucleotides complementary to the 5' receptor coding sequences have recently been used to generate cDNA libraries that include additional clones representing the 5' untranslated region(s) of c-fms mRNA, and our preliminary data suggest that other transcripts may be initiated from the same upstream region. At face value, then, the size of the c-fms locus may be greater than 58 kb. The identification of promoter/enhancer sequences within the c-fms gene and the characterization of trans-acting factors that govern CSF-1R transcription in human mononuclear phagocytes will be necessary prerequisites to defining the basis of receptor expression in malignant myeloblasts (see Addendum).

CSF-1 receptors on the surface of either normal monocytes or leukemic blasts were found to be more labile than other myeloid antigens routinely detected by flow cytometry (eg, CD13, CD14, CD33). Cryopreserved leukemic blasts had to be cultured overnight in medium lacking human CSF-1 to allow upregulation of receptors; the cells then had to be harvested and stained at 4°C in the presence of sodium azide to minimize subsequent receptor downmodulation. Aspects of sample manipulation during isolation and long-distance shipping may have contributed to the apparently smaller percentage of CSF-1-receptor-positive cases among adult patients. A more extensive survey will be necessary to determine whether there is a significant difference in CSF-1 receptor expression by myeloblasts from children and adults.

Others have identified c-fms mRNA in a somewhat higher percentage of leukemic cells from patients with AML by Northern blotting analysis5,27-29 or by in situ hybridization.27-28 Transcripts for CSF-1 were also identified in some leukemic samples, and some myeloid leukemic blasts coexpressed transcripts for both CSF-1 and its receptor, indicative of an autocrine mechanism for receptor activation in these cases.27-28 Persistent downmodulation of CSF-1 receptors was observed in fibroblast and macrophage cell lines engineered to express both CSF-1 and CSF-1R,21 suggesting that under such circumstances CSF-1R might not be detected by flow cytometry. We therefore believe that the percentage of receptor-positive cases recorded in our survey is likely to be an underestimate.

There was no obvious correlation between the presence of CSF-1R on leukemic blasts and treatment outcome for the patients studied; however, studies of larger numbers of uniformly treated patients with AML are needed to fully assess the potential clinical significance of our findings. As a participating laboratory for the Fourth International Conference on Human Leukocyte Differentiation Antigens, we have tested the workshop's panel of the independently developed myeloid-specific MoAbs for their ability to recognize epitopes of the human CSF-1R on our murine cell line engineered to express this receptor. All 165 MoAbs in this panel were negative for CSF-1R detection (R. A. Ashmun and A. T. look, unpublished data), indicating the uniqueness of our newly developed reagents. Aside from their utility as diagnostic reagents, MoAbs that bind epitopes in the extra-cellular ligand-binding domain of CSF-1R may have therapeutic applications. Preliminary evidence based on testing of mouse fibroblasts that functionally express the transduced human c-fms gene suggests that four of these antibodies recognize epitopes that interfere with ligand-receptor inter-
actions. It may therefore prove feasible to specifically affect the functional capabilities of normal mononuclear phagocytes or the growth potential of myeloid leukemias by judicious clinical use of these MoAbs, either alone or conjugated with other effector molecules.

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REFERENCES

15. Tamura T, Simon E, Neimann H, Snook GT, Bauer H: Gpl40† molecules expressed at the surface of cells transformed by the McDonough strain of feline sarcoma virus are phosphorylated in tyrosine and serine. Mol Cell Biol 6:4745, 1986
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37. Sutherland GR, Baker E, Callen DF, Campbell HD, Young IG, Sanderson CJ, Garson OM, Lopez AF, Vadas MA: Interleukin-5 is at Sq31 and is deleted in the 5q- syndrome. Blood 71:1180, 1988


44. Chen BD, Lin HS, Hsu 5: Tumor-promoting phorbol esters inhibit the binding of colony stimulating factor (CSF-1) to murine peritoneal exudate macrophages. J Cell Physiol 116:207, 1983

45. Roberts WM, Look AT, Roussel MF, Sherr CJ: Tandem linkage of human CSF-1 receptor (c-fms) and PDGF receptor genes. Cell 55:655, 1988
Monoclonal antibodies to the human CSF-1 receptor (c-fms proto-oncogene product) detect epitopes on normal mononuclear phagocytes and on human myeloid leukemic blast cells

RA Ashmun, AT Look, WM Roberts, MF Roussel, S Seremetis, M Ohtsuka and CJ Sherr