Expression of Human Colony-Stimulating Factor-1 (CSF-1) Receptor in Murine Pluripotent Hematopoietic NFS-60 Cells Induces Long-Term Proliferation in Response to CSF-1 Without Loss of Erythroid Differentiation Potential

By Roland P. Bourette, Guy Mouchiroud, Roland Ouazana, François Morlé, Jacqueline Godet, and Jean-Paul Blanchet

NFS-60 and FDCP-Mix cells are interleukin-3-dependent multipotent hematopoietic cells that can differentiate in vitro into mature myeloid and erythroid cells. Retrovirus-mediated transfer of the human colony-stimulating factor-1 (CSF-1) receptor gene (c-fms) enabled NFS-60 cells but not FDCP-Mix cells to proliferate in response to CSF-1. The phenotype of NFS-60 cells expressing the human CSF-1 receptor (CSF-1R) grown in CSF-1 did not grossly differ from that of original NFS-60 as assessed by cytochemical and surface markers. Importantly, these cells retained their erythroid potentiality. In contrast, a CSF-1-dependent variant of NFS-60, strongly expressing murine CSF-1R, differentiated into monocyte/macrophages upon CSF-1 stimulation and almost totally lost its erythroid potentiality. We also observed that NFS-60 but not FDCP-Mix cells could grow in response to stem cell factor (SCF), although both cell lines express relatively high amounts of SCF receptors. This suggests that SCF-R and CSF-1R signalling pathways share at least one component that may be missing or insufficiently expressed in FDCP-Mix cells. Taken together, these results suggest that human CSF-1R can use the SCF-R signalling pathway in murine multipotent cells and thereby favor self-renewal versus differentiation.

COLONY-STIMULATING factor-1 (CSF-1) is a 70- to 90-Kd homodimeric glycoprotein that stimulates proliferation, differentiation, and survival of cells belonging to the monocyte-macrophage lineage. Its effects are mediated by a single class of high-affinity receptors (CSF-1 receptor [CSF-1R]) that are expressed on monocytes, macrophages, and their committed bone marrow progenitors. CSF-1R, encoded by the c-fms proto-oncogene, is a member of subclass III of the tyrosine kinase receptor family, which also includes platelet-derived growth factor (PDGF) and stem cell factor (SCF) receptors. We and others have shown that ectopically expressed CSF-1R could induce CSF-1-dependent growth in fibroblasts and a number of murine factor-dependent hematopoietic cell lines, including myeloid FDC-P1 and 32D cells, and B-lymphoid cells, and mast cells. Interestingly, FDC-P1 cells transduced with human CSF-1R cDNA cannot differentiate in response to CSF-1, whereas CSF-1 can induce reversible mononuclear differentiation in FDC-P1 cells overexpressing murine CSF-1-R. This suggests that signalling pathways leading to proliferation and differentiation upon stimulation by CSF-1 are uncoupled in early myeloid cells and that human CSF-1R can favor proliferation versus differentiation in these cells. Given this assumption, ectopically expressed human CSF-1R would confer CSF-1-dependent growth to multipotent hematopoietic cells without altering their differentiation potential.

To address this question, we have investigated the effects of human CSF-1R when expressed in murine interleukin-3 (IL-3)-dependent multipotent NFS-60 and FDCP-Mix cells. The NFS-60 cell line, derived from leukemia cells after in vivo infection with ecotropic Cas-Br-M murine leukemia virus, can differentiate into neutrophils and macrophages in the presence of IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF), whereas erythropoietin (Epo) induces both erythroid and nonerythroid colony formation. Moreover, single-cell transfer experiments have shown that a single NFS-60 cell could develop into these different lineages, thus suggesting that NFS-60 is a pluripotential cell line. FDCP-Mix cells, obtained from a long-term bone marrow cell culture infected with src-Moloney murine leukemia virus, can differentiate into mature neutrophils and monocytes in response to IL-3 and into mixed erythroid/myeloid colonies in the presence of both Epo and IL-3. In the following studies, we specifically asked (1) if CSF-1 could substitute for IL-3 in sustaining long-term proliferation of multipotent hematopoietic cells expressing human CSF-1R, and (2) if CSF-1-dependent proliferation of such cells would alter their differentiation potentialities, particularly erythroid differentiation, because erythropoiesis and monocytic differentiation are currently considered to be mutually exclusive.

We report here that human CSF-1R confers CSF-1 responsiveness and long-lasting proliferation to NFS-60 cells but not to FDCP-Mix cells. In addition, we show that NFS-60, but not FDCP-Mix, cells can grow in response to SCF, although both cell lines express relatively high amounts of SCF receptors (SCF-R). A CSF-1-dependent cell line (N/fms/CSF-1) was derived from NFS-60 cells transduced with the human CSF-1R cDNA. Analysis of N/fms/CSF-1 cells and seven derived clones showed that CSF-1 did not globally alter the cell phenotype as assessed by cytochemical studies and analysis of a number of surface markers. Moreover, each of these cell lines retained their ability to form erythropoietic colonies in response to Epo in semi-solid cultures.

MATERIALS AND METHODS

Growth factors. Purified recombinant murine IL-3 (mIL-3) and purified recombinant human Epo (rhEpo) were purchased from...
Genzyme (Boston, MA) and Boehringer Mannheim (Mannheim, Germany), respectively. Purified recombinant murine SCF (rmSCF) was a gift of Dr. S. Gillis (Inmunex, Seattle, WA). The source of recombinant human CSF-1 (rhCSF-1) was a medium conditioned by COS-7 cells transiently transfected by the p3ACSFRI plasmid (gift of Dr. G. Wong, Genetics Institute, Cambridge, MA) that carries human CSF-1 cDNA.11 This conditioned medium, called COS-CSF-1 CM, contained approximately 40,000 CSF U/mL as assessed in cultures of murine bone marrow cells.11 Purified rhCSF-1 was obtained from British Biotechnology (Abingdon, UK).

Cell lines. NFS-60 cells, a gift of Dr. S. Sealand (Scherin-Plough, Dardilly, France), were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Boehringer Mannheim) supplemented with 10% fetal calf serum (FCS, Biosys, Compiegne, France). 5% WEHI conditioned medium (WEHI-CM) as a source of IL-3, and antibiotics. FDCP-Mix cells, kindly provided by Dr. E. Spenceur (Manchester, UK), were maintained in Iscove’s modified Dulbecco’s medium (IMDM; Boehringer Mannheim) supplemented with 20% horse serum (HoS; Biosys), 15% WEHI-CM, and antibiotics. Culture medium was changed when cell density reached 5 x 10^5 cells/mL, every 2 or 3 days.

The NFS-60/MAC cell line was obtained as follows. NFS-60 cells were seeded in DMEM-10% FCS supplemented with 5% COS-CSF-1 CM as the sole growth factor. Under these conditions, all cells apparently died, but after 2 weeks, a population of semi-adherent cells, heterogeneous in size, began to proliferate. This cell population was established as a CSF-1-dependent cell line, called NFS-60/MAC. As shown in Results, the majority of NFS-60/MAC cells is composed of cells exhibiting a monococyte-macrophage-like phenotype.

Retroviral vectors and infection. Recombinant ecotropic GP + E-86 packaging cell lines producing NTK-c-fms or N2 retroviruses were provided by Dr. T. von Rüden (IMP, Vienna, Austria).11 In the NTK-c-fms vector, the neomycin resistance gene (neo) is expressed from the viral long terminal repeat (LTR), whereas the human c-fms cDNA is expressed under transcriptional control of Herpes simplex thymidine kinase (TK) gene promoter. N2 vector, carrying only neo gene, was used as control.

Amphotropic packaging cell lines GP + envAm 1217 (a gift of Dr. A. Bank, Columbia, NY) were infected with ecotropic NTK-c-fms or N2 viruses; 24 hours after infection, cells were plated in medium containing 0.8 mg/mL G418 (GHBCO, Grand Island, NY) for 10 days. NFS-60 or FDCP-Mix cells were then infected by incubating 1 to 4 x 10^5 target cells for 48 hours in a mixture of 5 mL of viral supernatant, 5 mL of IL-3-containing culture medium, and 8 ng/mL polybrene (Aldrich, Steinheim, Germany). Neo’ cells were then selected in complete medium containing 0.8 mg/mL G418 for 15 days.

DNA and RNA analysis. High molecular weight DNAs were prepared using standard methods.18 The integrity of the proviral insert was evaluated after complete digestion with Kpn I, which cuts once within each proviral LTR; the integration site was studied using HindIII, whose restriction site is absent from the viral genome and which thus generates fragments characteristic of each integration site. After separation on 0.8% agarose gels, DNA fragments were transferred onto a nylon filter and hybridized to a 32P-labeled c-fms cDNA probe.

Poly(A) RNA was extracted from 10^7 cells using oligo(dT) cellulose and then fractionated on formamide/agarose gels (2 to 3 ìg for each sample), according to standard protocols.18 After transfer to nylon membranes, the blots were hybridized to 32P-labeled c-fms probe.

Antibodies and immunofluorescence labeling. Rat monoclonal antibodies (MoAbs) to human CSF-1-R were to be referred to as Ab-1 (clone 2-4A5-4; Oncogene Science, Manhasset, NY) and Ab-2 (clone 3-4A4-E4; kindly provided by Dr. C.J. Sherr, St. Jude Children’s Research Hospital, Memphis, TN).26 These MoAbs do not detect the murine form of the CSF-1-R. Rabbit antibodies to murine CSF-1-R2 were a gift of Dr. L.R. Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA). Rat MoAb to the murine CSF-R/c-kit protein (ACK2)27 was a gift of Dr. S.-I. Nishikawa (Kumamoto University Medical School, Kumamoto, Japan). Antibodies directed against hematopoietic lineage-specific surface antigens were as follows: F4/80 (a gift of Dr. G. Milan, Institut Pasteur, Paris, France), which detects a macrophage-specific marker21; M1/70 (Biosys), which detects the Mac-1 antigen on granulocytes and macrophages25; RA3-6B2 (Caltag, San Francisco, CA), which detects the B220/Ly-5 antigen on pre-B and B cells24; and RB6-8C5 (Caltag), which detects an antigen found predominantly on neutrophils.26 The mouse anti-Thy-1.2 MoAb (clone S-88), which detects a marker on T cells and early myeloid cells,26 was purchased from Cedarlane (Hornby, Canada).

For immunofluorescence staining, antibodies were diluted to predetermined concentrations in phosphate-buffered saline (PBS) containing 5% FCS and 0.2% sodium azide (PBS-FCSNa3) supplemented with 40 ìg/mL of Igs from the same species as the relevant secondary antibodies. Cells (5 x 10^6) were incubated with primary antibodies for 45 minutes on ice and then washed with PBS-FCSNa3 and incubated in the same conditions with fluorescein isothiocyanate (FITC)-conjugated second-antibodies diluted in PBS-FCSNa3. These secondary antibodies were goat anti-rat IgG (Sigma, St. Louis, MO), goat antirabbit IgG (Sigma), or rabbit anti-mouse Igs (Dako, Glostrup, Denmark). After two washings in PBS-FCSNa3, cells were resuspended in the same medium with 1% formalin and analyzed on a FACStar Plus apparatus (Becton Dickinson, Palo Alto, CA).

Morphologic and histochemical analysis. Cells either from liquid cultures or from colonies grown in methylcellulose cultures were cytospun onto glass slides, air-dried, and stained with May-Grünwald-Giemsa (Sigma). Before histochemical analysis, cells were fixed in citrate-acetone-formaldehyde solution, examined for naphtol AS-D chloroacetate esterase and a-naphthyl acetate esterase using staining kits (Sigma), and counterstained with hematoxylin (Sigma).

Methylcellulose cultures. NFS-60-derived cell lines (250 to 2,500 cells/mL) were plated in DMEM supplemented with 10% FCS, 1% bovine serum albumin (BSA; Boehringer-Mannheim), 7.5 x 10^4 mol/L 2-mercaptoethanol (Sigma), antibiotics, 1% methylcellulose (Fluka A.G., Buchs, Switzerland), and growth factors as specified. FDCP-Mix–derived cell lines (1,250 to 12,500 cells/mL) were plated in IMDM supplemented with 24% HoS, 1% BSA, 7.5 x 10^-5 mol/L 2-mercaptoethanol, antibiotics, 1% methylcellulose, and growth factors as specified. Cultures (400 ìL) were performed in 24-well plates (Costar, Cambridge, MA) and incubated at 37°C and 5% CO2 in a fully humidified atmosphere; 10 to 15 days later, colonies were counted using an inverted microscope.

Proliferation assay. Proliferation and viability were assessed as described by Mosman.27 Briefly, cells were washed twice and then plated in culture medium supplemented with various concentrations of growth factors as specified. Cultures (100 ìL) were set up in duplicate in 96-well microtitre plates (Nunc, Roskilde, Denmark). Plates were incubated for 48 or 72 hours at 37°C and 5% CO2 in a fully humidified atmosphere. Proliferation was then monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay.27

RESULTS

Expression of human CSF-1R on NFS-60 cells leads to CSF-1-dependent proliferation. NFS-60 cells were infected for 48 hours with supernatants of amphotropic N2 or NTK-c-fms virus-producing cells, selected in G418 (0.8 mg/mL)
and then amplified in the presence of IL-3. G418' NFS-60 cells were called N/N2 and N/fms, respectively. As expected, human c-fms mRNA could be detected in N/fms cells as three transcripts of 8.0, 7.5, and 3.7 kb corresponding to genomic and subgenomic transcripts initiated from the 5' viral LTR and internal transcript initiated by TK promoter, respectively (data not shown). Expression of human CSF-1R on N/fms cells was shown by flow cytometry after labeling with anti-hCSF-1R MoAbs (Fig 1A).

After cultivating for 8 days in liquid cultures supplemented with rhCSF-1 alone, N/fms cells, but not N/N2 cells, readily proliferated (Fig 1B) and gave rise to a CSF-1–dependent cell line called N/fms/CSF-1. The doubling time of N/fms/CSF-1 cells was about 22 hours in CSF-1 versus 12 hours in IL3; no factor-independent growth of these cells could be observed (not shown). Flow cytometry analysis of N/fms/CSF-1 cells showed a dramatic increase in human CSF-1R expression on their cell surface as compared with N/fms cells (Fig 1A). To determine whether the N/fms/CSF-1 cell population resulted from selection of a few N/fms clones, provirus integration sites were determined for 22 independent N/fms/CSF-1 clones. Half of them exhibited different insertion patterns (not shown), which enabled us to conclude that our N/fms/CSF-1 population is polyclonal.

The function of CSF-1 is to induce proliferation and differentiation of monocyte progenitors. Because N/fms/CSF-1 cells proliferated in response to CSF-1, we questioned whether long-term cultivation of these cells would result in their commitment towards monocytic lineage. Thus, the following experiments were undertaken to determine the effects of long-term CSF-1–dependent proliferation (1 to 3 months) of N/fms/CSF-1 cells on their phenotype (morphology and expression of myeloid markers), their growth properties (clonal growth in response to IL-3 and CSF-1), and their ability to form erythroid colonies in response to Epo. In these experiments, control cells were N/N2 cells, representative of undifferentiated NFS-60 cells, and NFSdO/MAC cells, representative of NFS-60 cells that have differentiated along the monocytic lineage (see below).

Morphologic and flow cytometry analysis of NFS-60–derived cell lines. Cell lines were stained using the May-Grunwald-Giemsa reagent. We then observed that N/fms/CSF-1 maintained in CSF-1 for 1 month exhibited a blastic phenotype, similar to N/N2 and N/fms cells. In addition, a-naphthyl acetate esterase, specific for monocytes, was not expressed in N/N2, N/fms, or N/fms/CSF-1 cells. In contrast, NFS-60/MAC cells showed a macrophage-like morphology in association with a high level of α-naphthyl acetate esterase activity (not shown).

Flow cytometry analysis of myeloid cell surface markers showed that N/N2 and N/fms cells exhibited similar expression patterns (not shown). A comparative study of N/N2, N/fms/CSF-1, and NFS-60/MAC cells is shown in Fig 2. High levels of early marker Thy-1 expression were found on N/N2 and N/fms/CSF-1 cells, whereas F4/80 and 8C5 antigens could not be detected on either cell line. Even the expression levels of Mac-1 antigen and murine CSF-1R were found to be clearly increased on N/fms/CSF-1 cells as compared with N/N2 cells. The highest expression level of the latter markers was found on NFS-60/MAC cells, which, in addition, became strongly reactive with anti-F4/80 and almost negative for Thy-1 expression.

Seven of the clones derived from N/fms/CSF-1 cells (see above) were cultivated for 2 months either in IL-3 or human CSF-1. No overall significant increase in marker expression could be shown for cells maintained in CSF-1, as compared with IL-3 cultures, except for Mac-1 antigen, which was significantly increased in the presence of CSF-1. When clones were considered individually, only two showed additional...
Fig 2. Flow cytometry analysis of expression of cell surface markers on NFS-60-derived cell lines. Surface antigen expression [——] on control N/N2 cells maintained in IL-3 was compared with expression on N/fms/CSF-1 and NFS-60/MAC cells maintained in CSF-1. The cells were stained with anti-Thy-1 antibodies, 8C5 MoAbs, anti-murine c-fms antibodies, anti-Mac-1 MoAbs, and F4/80 MoAbs; background fluorescence (---) was assessed using rat B220 antibody.
changes: clone 1, with a weak expression of F4/80 and a clear increase in murine CSF-1R expression; and clone 8, exhibiting high expression of F4/80 and murine CSF-1R associated with a reduced expression of Thy-1 antigen (not shown).

We concluded from these studies that N/fms/CSF-1 cells have not differentiated along monocyte or granulocyte lineages, although an increase in expression of early monocyte markers such as Mac-1 and murine CSF-1R could be seen.

**Growth properties of NFS-60 cells expressing the human CSF-1R**. Growth properties of NFS-60-derived cell lines were assessed in methylcellulose cultures (Table 1). In the absence of any added growth factors, N/N2, N/fms, and N/fms/CSF-1 cells did not proliferate. In contrast, about 8.5% of NFS-60/MAC cells could grow under these conditions. Because these cells exhibit macrophage-like features, we suggest that they might produce CSF-1 and proliferate in an autocrine manner.

No significant differences in the growth response to rmIL-3 were observed between N/N2, N/fms, and N/fms/CSF-1 cells that developed large and compact colonies (Fig 3A), composed of undifferentiated blast cells with rare mature monocytes and granulocytes. In contrast, IL-3 responsiveness was significantly decreased in NFS-60/MAC cells, with some colonies being diffuse and composed of macrophages (not shown).

N/N2 cells responded weakly to CSF-1 (0.6%), such as the parental cells; this finding is at variance with a previous report.11 A significantly higher proportion of N/fms cells formed colonies in response to rhCSF-1 (1% to 2%), but still very few as compared with their response to IL-3. Interestingly, cloning efficiencies of N/fms/CSF-1 and NFS-60/MAC were similar in the presence of CSF-1; however, colonies observed with N/fms/CSF-1 were compact (Fig 3B) and essentially contained undifferentiated cells, whereas colonies observed with NFS-60/MAC cells were diffuse (Fig 3C) and mainly composed of monocytes and macrophages. When the growth inhibitory anti-human CSF-1R MoAb Ab-1 was introduced at 5 μg/mL final concentration into methylcellulose cultures, CSF-1-dependent clonal growth of N/fms/CSF-1 cells was abolished (4% ± 2% with Ab-1 v 38% ± 7% without Ab-1), but not their IL-3-dependent clonal growth (54% ± 4% with Ab-1 v 60% ± 4% without Ab-1).

**Erythroid differentiation of NFS-60-derived cell lines.** Differentiation potentials of our NFS-60-derived cell lines were monitored using a clonal methylcellulose assay performed in the presence of Epo. N/N2, N/fms, and N/fms/CSF-1 cells responded similarly to rhEpo (Table 2). About 50% to 60% of total colonies were well hemoglobinized (Fig 3D), the remainder being composed of apparently undifferentiated colonies, referred to as nonerythroid colonies. Response of these cells to Epo was found to be much higher than originally described, with a total of 17 ± 2 colonies per 100 plated cells in our experiments versus 59 ± 4 per 10⁵ cells in Hara et al.15 Capacity of N/fms/CSF-1 cells to form erythroid colonies in response to Epo was still observed after maintaining the cells for 3 months in human CSF-1 (Fig 3D).

In addition, N/fms/CSF-1-derived subclones developed erythroid colonies in response to rhEpo at a similar rate regardless of whether they were maintained in CSF-1 or IL-3 (not shown). In contrast, NFS-60/MAC cells responded weakly to Epo, the frequency of erythroid colonies dramatically decreasing to 0.1%; they no longer exhibited erythroid differentiation ability after 2 months in CSF-1 (not shown).

We concluded from these experiments that human CSF-1R can allow proliferation of NFS-60 cells in response to CSF-1 for 3 months without altering their erythroid differentiation potential.

**CSF-1 is unable to support the growth of FDCP-Mix cells expressing human CSF-1R.** F-Mix/N2 and F-Mix/fms cells were obtained from FDCP-Mix cells infected with supernatants of amphotropic N2 or NTK-c-fms virus-producing cells, respectively, and then selected in G418 (0.8 mg/mL). F-Mix/fms cells, but not F-Mix/N2 cells, expressed human CSF-1R (Fig 4), with an expression level apparently similar to that of N/fms cells. However, F-Mix/fms cells could not grow in response to human CSF-1, nor could they survive longer than 3 days in rhCSF-1. To determine whether the response to rhCSF-1 could be restricted to a minute number of cells, semisolid cultures were performed. No clonal response to rhCSF-1 could be observed even when 10⁴ cells were plated.

To assess functional expression of the human CSF-1R on FDCP-Mix cells, F-Mix/N2 and F-Mix/fms cells were cultivated for 3 days in the presence of 25 U/mL rmIL-3 and increasing concentrations of purified rhCSF-1. Whereas rhCSF-1 had no effect on IL-3-induced proliferation of F-Mix/N2 cells, it significantly (P < .05) increased the response of F-Mix/fms cells to rmIL-3 (Fig 5). In addition, this stimulatory effect could be abolished when Ab-1 antibody was introduced into the cultures (Fig 5).

**SCF-R expression on F-Mix/fms and N/fms cells and proliferative response to rmSCF.** Several studies have suggested that c-kit/SCF-R is structurally and functionally related to CSF-1R. Recently, Dubreuil et al.13 have shown that murine c-fms gene could complement the mitogenic defect in mast cells derived from mutant W (SCF-R-deficient) mice. Moreover, the signal transduction pathways of these two receptors might share common substrates.24 Because N/fms cells responded to CSF-1 and F-Mix/fms cells did not, we examined the attractive hypothesis that these cells also differ in their response to SCF. Before this study, we have monitored SCF-R expression on F-Mix/fms and N/fms cells by flow cytometry using specific rat MoAb ACK2.21 Both F-Mix/fms (Fig 6A) and N/fms cells (Fig 6B) expressed high levels of SCF-R, with

### Table 1. Clonal Growth of Different NFS-60-Derived Cell Lines in Response to IL-3 and CSF-1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No GF</th>
<th>63.3 ± 7.9</th>
<th>61.0 ± 1.6</th>
<th>68.0 ± 4.1</th>
<th>43.7 ± 3.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/N2</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N/fms</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N/fms/CSF-1</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFS-60/MAC</td>
<td>8.5 ± 2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells (100 to 1,000) were seeded in 1% methylcellulose-containing medium supplemented with growth factors as specified. Macroscopic colonies of more than 100 cells were enumerated 10 to 15 days later. Results are means ± SD of three independent experiments.
a higher expression on N/fms cells. Similar results were obtained with F-Mix/N2 and N/N2 cells, respectively (data not shown).

We then investigated short-term proliferation of these cells in response to rmSCF. F-Mix/fms cells did not proliferate in response to rmSCF even at concentrations as high as 200 ng/mL (Fig 7A). However, rmSCF (200 ng/mL) could potentiate effects of infraoptimal rmIL-3 concentrations (Fig 7A and Dr T.M. Dexter, Manchester, UK, personal communication, October 1991), suggesting that SCF signalling pathway partly functions in FDCP-Mix cells, but is not able to sustain a complete proliferative response (similar results were obtained with F-Mix/N2 cells, data not shown). With regards to N/fms cells, rmSCF stimulated their proliferation, but to a lesser extent than rmIL-3 (Fig 7B); similar results were obtained with N/N2 cells (not shown).

DISCUSSION

FDCP-Mix and NFS-60 cells are multipotent hematopoietic cells growing indefinitely in the presence of IL-3. We report here the effects of human CSF-1R expression in these two cell lines. We were specifically interested in determining if human CSF-1R could provide multipotential cells with the capacity for extensive self-renewal in the presence of CSF-1.

At variance with a previous report,1 it we found that NFS-60 cells proliferated slightly in methylcellulose cultures sup-
implemented with CSF-1, indicating that the murine CSF-1R was expressed on the cells; this was confirmed by flow cytometry (Fig 2). We also observed that no meaningful survival nor proliferation of NFS-60 cells could be observed in short-term liquid cultures supplemented with CSF-1 alone (Fig 1B). In contrast, after transfer of human c-fms, a CSF-1-dependent population (N/fms/CSF-1 cells) could be rapidly derived from NFS-60 cells. Because expression of the murine CSF-1R was also increased on these cells (Fig 2) and because human CSF-1 binds to both receptors, we asked which of the human or murine CSF-1Rs mediated the mitogenic signal. As proliferation of N/fms/CSF-1 cells in response to rhCSF-1 was abolished in the presence of the growth inhibitory MoAb Ab-1, specific to the human CSF-1R, we conclude that the proliferation signal induced by rhCSF-1 in N/fms/CSF-1 cells only involves the transfected human CSF-1R.

FDCP-Mix cells transduced with the human c-fms cDNA (F-Mix/fms cells) express human CSF-1R, at a level similar to that of NFS-60 cells, but do not proliferate in response to rhCSF-1. Seeding up to $10^5$ F-Mix/fms cells into liquid or methylcellulose cultures did not result in any CSF-1-dependent proliferation. However, rhCSF-1 could potentiate the stimulatory activity of a suboptimal IL-3 concentration on the growth of F-Mix/fms cells. This effect of CSF-1 was mediated by the human CSF-1R because it could be abolished by the Ab-1 MoAb. We conclude that the human CSF-1R
can be functionally expressed on FDCP-Mix cells, but that these cells lack at least one component essential for the transduction of a complete mitogenic signal from the human CSF-1R.

Ectopically expressed human CSF-1R confers CSF-1 responsiveness to a number of IL-3–dependent cell lines.6-11 The hypothesis that human CSF-1R can use the signalling pathway evoked from the IL-3R can be ruled out because of our finding that FDCP-Mix cells, which are strictly dependent on IL-3 for growth, do not respond to CSF-1 after transfer of the c-fms gene. Moreover, Kinashi et al29 have recently shown that expression of human CSF-1R on murine multipotent IL-3–dependent Lyd9 cells did not induce responsiveness to CSF-1, nor to their GM-CSF or granulocyte-CSF (G-CSF) derivatives. Taken together, these data show that human CSF-1 R does not use signalling pathways specific for IL-3, GM-CSF, and G-CSF when expressed in a factor-dependent cell line. We have shown here that NFS-60 cells that express SCF-R and proliferate in response to SCF are also able to respond to CSF-1 after transduction of human CSF-1R. In contrast, FDCP-Mix cells that express a high number of SCF-R, but do not proliferate in response to SCF, do not proliferate in CSF-1 when expressing human CSF-1R (this study). In addition, human CSF-1R confers CSF-1 responsiveness to (1) FDC-P1 cells that express SCF-R and proliferate in response to SCF (our unpublished data and Williams et al69) and (2) 32D cells that do not express SCF-R, but could respond to SCF after the transfer of murine c-kit gene (Dr S. Lyman, Immunex Corp, Seattle, WA, personal communication, February 1992). Thus, a good correlation appears between the ability of a murine myeloid cell to respond to CSF-1 after transfer of human CSF-1R and its capacity to proliferate in response to SCF. The CSF-1R and SCF-R might use, at least partially, a common signal transduction pathway for mediating their proliferative effects. If this signalling pathway is absent or incomplete, ectopic expression of these receptors could not induce a growth response.

Because CSF-1R normally transduces both mitogenic and differentiating signals in bone marrow cells, we asked whether long-term CSF-1–dependent growth would alter the differentiation of NFS-60 cells. N/fms/CSF-1 cells cultivated for 1 month in CSF-1 were not affected in their immature phenotype. They showed a blast morphology, did not express nonspecific esterases, were highly positive for the early marker Thy-1 and negative for the monocytic marker F4/80, and could differentiate into the erythroid lineage in response to Epo. This phenotype was compared with those of two control cell lines. First, N/N2 cells, which only express the neo gene, were used as undifferentiated control cells. We have shown here that N/fms/CSF-1 cells and seven derived subclones have acquired CSF-1–dependent growth while exhibiting essentially the same characteristics as N/N2 cells. Second, NFS-

### Table 2. Responsiveness of Different NFS-60–Derived Cell Lines to Epo in Semi-Solid Culture

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Erythroid Colonies</th>
<th>Nonerythroid Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/N2</td>
<td>9.7 ± 1.7</td>
<td>7.7 ± 2.3</td>
</tr>
<tr>
<td>N/fms</td>
<td>11.0 ± 6.0</td>
<td>9.0 ± 5.4</td>
</tr>
<tr>
<td>N/fms/CSF-1</td>
<td>8.7 ± 1.7</td>
<td>5.7 ± 2.5</td>
</tr>
<tr>
<td>NFS-60/MAC</td>
<td>0.1 ± 0.1</td>
<td>5.4 ± 2.4</td>
</tr>
</tbody>
</table>

Cells (100 to 1,000) were seeded in 1% methylcellulose-containing medium supplemented with growth factors as specified. Macrocscopic colonies of more than 100 cells were enumerated 10 to 15 days later. Results are means ± SD of three independent experiments.
TRANSFER OF CSF-1R INTO PLURIPOTENT CELLS

60/MAC cells were derived from NFS-60 cells after selecting in CSF-1. These cells express a high level of murine CSF-1R and are clearly committed to the monocytic lineage. For these reasons, they were used as control cells for monocytic differentiation of NFS-60 cells. Although N/fms/CSF-1R cells and their subclones showed an increased expression of early monocytic Mac-1 marker and murine CSF-1R, we could not find any evidence that they have embarked upon monocytic differentiation as shown by the expression patterns of F4/80 (negative) and Thy-1 (positive) antigens. In addition, NFS-60/MAC cells could not give rise to erythroid colonies in response to Epo, whereas N/fms/CSF-1R cells did. In conclusion, N/fms/CSF-1R cells appeared to be fixed in their potentialities and could not be modulated by either IL-3 or CSF-1.

It appears from our study that N/fms/CSF-1R cells and NFS-60/MAC cells, both CSF-1-dependent for their proliferation, have very contrasting phenotypes. Latter cells express murine CSF-1 and have a differentiated phenotype, whereas N/fms/CSF-1R cells use human CSF-1R for growth in CSF-1 and are undifferentiated. This would suggest that human and murine CSF-1R might have different effects when expressed in NFS-60 cells. A similar situation has been previously reported with FDC-P1 cells; murine CSF-1R expressed from a retroviral vector induces reversible monocytic differentiation of FDC-P1 cells in response to CSF-1, whereas FDC-P1 cells expressing the human CSF-1R can become CSF-1-dependent without differentiating. Recently, we have obtained NFS-60 cells expressing the murine CSF-1R cDNA from a retroviral vector (a gift of Dr L.R. Rohrschneider). These cells could give rise to a CSF-1-dependent population. However, in contrast to N/fms/CSF-1R cells, these cells exhibited a relatively differentiated phenotype and, interestingly, a dramatic decrease (by a 50-fold factor) in their erythroid response to Epo as compared with control cells (unpublished data). Thus, we propose that in NFS-60 cells (this study) and FDC-P1...
human CSF-1R only provides the cells with a proliferative stimulus, whereas the murine CSF-1R might deliver both proliferative and differentiating signals. Thus, two different biochemical pathways for proliferation or differentiation could be assumed (as was recently demonstrated for the Epo receptor). If the human CSF-1R only provides a proliferative signal to cells by using (totally or partially) the SCF-R signalling pathway, its transfer into early hematopoietic progenitors (SCF-sensitive) would be an attractive method for their expansion in vitro upon CSF-1 stimulation.

ACKNOWLEDGMENT

We thank Dr Thomas von Rüden (IMP, Vienna, Austria) for the gift of packaging cell lines, Drs Bernard Verrier and Bernard Mandran (CNRS-Bioméreux, Lyon) for P3 facility, Dr Steven Gillis (Immunex Corp., Seattle, WA) for providing us with murine recombinant SCF, Dr Gérard Lizard for helpful assistance with flow cytometry, and Dr Tim Greenland for critical review of the manuscript.

REFERENCES

Expression of human colony-stimulating factor-1 (CSF-1) receptor in murine pluripotent hematopoietic NFS-60 cells induces long-term proliferation in response to CSF-1 without loss of erythroid differentiation potential

RP Bourette, G Mouchiroud, R Ouazana, F Morle, J Godet and JP Blanchet