Macrophage Colony-Stimulating Factor (CSF-1) Gene Expression in Human T-Lymphocyte Clones

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Macrophage colony stimulating factor (CSF-1) is one of several cytokines that control the differentiation, survival, and proliferation of monocytes and macrophages. A set of 11 human T-cell clones, chosen for their phenotypic diversity, were tested for their ability to express CSF-1 mRNA. After 5 hours of stimulation with phorbol myristate acetate (PMA) + calcium ionophore (Cal), all T-cell clones expressed a major 4-kb transcript, a less abundant 2-kb transcript, and several other minor species. This pattern of expression is typical for CSF-1 mRNAs. Furthermore, of the two alloreactive T-cell clones analyzed, only one showed a definitive message for CSF-1 on specific antigenic stimulation, with both delayed kinetics and less efficiency. Both conditions of stimulation induced the release of CSF-1 protein by T cells in the culture medium. Together, these findings demonstrate for the first time that normal T cells are able to produce CSF-1, previous reports being limited to two cases of tumoral cells of the T-cell lineage.

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MATERIAL AND METHODS

Cells. A panel of 11 human T-lymphocyte clones from different origins were used for this study. Five of them were derived from T cells infiltrating skin during acute graft-versus-host disease (GVHD) (Donor A), two of them were obtained from the PBLs of a patient with large granular lymphocyte disorder (LGLD) (Donor B), and four alloreactive T-lymphocytes clones (ATLC) from a donor C were derived from mononucleated cells invading an irreversibly rejected human kidney allograft. The monoclonality of all these cultures, cloned by the limiting dilution technique, has been previously confirmed by analyses of T-cell receptor (TCR) genes rearrangements (data not shown).

The phenotypic and functional characteristics of these clones are given in Table 1. They exhibit a wide range of surface marker phenotype (CD4^+CD8^-, CD4^+CD8^+, CD4^-CD8^-, CD4^-CD8^-; aβ or γδ TCR; cytotoxic or not), and are considered to be representative of the different T-lymphocyte subpopulations.

Culture conditions and cell stimulations. Because the antigens recognized by these cells are not known, the GVHD and LGLD T-lymphocyte cultures were maintained in the presence of recombinant interleukin-2 (rIL-2) under polyclonal activation: 10^5 to 5 x 10^6 cells/well were plated in microtiter plates ( Falcon, Grenoble, France) in the presence of 5 x 10^4 irradiated (30 Gy) alloreactive PBLs and 5 x 10^5 cells from an irradiated (30 Gy) B-lymphoblastoid cell line (BLCL), in RPMI 1640 supplemented with 10% human serum, pure rIL-2 (1 nmol/L) (Biogen, Geneva, Switzerland), indomethacin (1 µmol/L) (Sigma, St Louis, MO), and leucoagglutinin (1 µmol/L) (Pharmacia, Upsala, Sweden). ATLC cultures were maintained by weekly addition of irradiated (30 Gy) kidney-donor-derived BLCL (D-BLCL) at a 1:1 ratio in RPMI 1640 supplemented with 10% human serum and pure rIL-2 (1 nmol/L).

Responding cells (5 x 10^5/mL) in complete medium were stimulated either with 20 ng/mL Phorbol ester (PMA; Sigma, ref. 81-39) plus 2 nmol/L Calcium Ionophore, A23187 (Cal; Sigma, ref. C75-22) or, for ATLC cultures, with irradiated D-BLCL (30 Gy) at a ratio of 1:1, after 6 to 8 days of culture, at which time irradiated allogeneic stimulator cells were absent from cultures. Cells and their media were then harvested at different points of the time course for RNA blot and CSF-1 protein release analyses.

For CSF-1 production per day, cells from T-lymphocyte clone 2F7 were washed twice 7 days after antigenic stimulation and seeded at 0.5 x 10^6 cells/mL with irradiated (10,000 rads) D-BLCL (2.5 x 10^5/mL) in RPMI medium supplemented with recombinant human IL-2 (rhu IL-2) (1 nmol/L) and 10% human serum (identified as culture medium). At day 4, cultures were diluted 1 to
4 with fresh culture medium to prevent cell death because of medium exhaustion. Each day the cultures were washed twice, reseeded in fresh culture medium at 0.5 \times 10^6 cells/mL, and further incubated for 24 hours. The supernatant was stored at -20°C for further CSF-1 production per day analysis.

**RNA blot analysis.** Total cellular RNA was isolated from 30 \times 10^6 cells by using the guanidium isothiocyanate lysis procedure followed by cesium chloride density gradient centrifugation. Equal amounts of total RNA (0.015 mg as determined at 260-nm wavelength) were denatured for 15 minutes at 60°C in electrophoresis buffer (20 mmol/L morpholinopropionate sulfoonic acid, 5 mmol/L sodium acetate, 1 mmol/L EDTA) containing 6% formaldehyde (vol/vol) and 50% formamide (vol/vol), quick chilled on ice and then size-fractionated by electrophoresis through 1% agarose gel containing 6% formaldehyde. Blotting was performed on Gene screen plus filter (NEN, Boston, MA) according to the supplier's instructions. After baking at 80°C for 2 hours and prehybridization, filters were hybridized for 20 hours at 42°C with \( ^{32}P \)-labeled probe. Filters were washed twice for 10 minutes at room temperature and then once for 10 minutes at 65°C in x-ray film exposure. RNA blot analysis was done using bone marrow from 8- to 10-week-old Balb/c mice. Cells 2 \times 10^6 were plated in 0.1-mL cultures in microtiter wells in Iscoves medium containing 0.44 fmol.

**Murine bone marrow colony assays.** Murine bone marrow assays were performed as previously described, using bone marrow from femurs of 8- to 10-week-old Balb/c mice. Cells 2 \times 10^6 were plated in 0.1-mL cultures in microtiter wells in Iscoves medium containing 1.2% methylcellulose (Dow Chemical Company, Woburn, MA), 30% heat-inactivated fetal calf serum (Hazelton, Lanexa, KS), 1% BSA (GIBCO, Grand Island, NY), and either 10% or 2% conditioned medium. Each point was assayed in triplicate. Colonies were counted through an inverted microscope on day 7, and were considered to be macrophage colonies on the basis of their morphology.

Alternatively, colony assays were performed in collagen gels as described. After 7 days of culture, the gels were dried, fixed, and stained with May-Grünwald-Giemsa or butyrate esterases for identification of monocytic colonies.

**RESULTS**

Expression of CSF-1 transcripts in human T cells exposed to phorbol ester + calcium ionophore or to antigen stimulation. Total RNA extracted from CD4^-CD8^- γδ TCR cell clones from LGLD, from CD4^+CD8^- and CD4^-CD8^+ αβ TCR cell clones from GVHD (Fig 1) and from CD4^+CD8^-, CD4^-CD8^-, and CD4^+CD8^+ αβ TCR ATLCs (Figs 2 and 3) were analyzed by RNA blots. The RNA blot analysis showed significant amounts of M-CSF messages in total cellular RNA isolated from the T cell clones 5 or 6 hours after stimulation with PMA + Cal. All cell clones expressed (Fig 2) a major transcript surrounding 6 kb in size, a less abundant message of small size around 2 kb, and several other minor mRNA species.

From Fig 3, it can be seen that hybridization of mRNA from specific-antigen-stimulated 4W ATLC, with the M-CSF-specific cDNA probe, failed to show any significant induction of the expression of M-CSF gene. This result is in contrast to that obtained with the 2F7 ATLC, where the induction of multiple M-CSF transcripts were evident 11 hours after antigenic challenge. In this last case, despite the signal being smaller compared with the previous situation
where PMA + CaI were used to activate T cells, both the 4-kb and the 2-kb mRNAs were clearly induced by such antigenic stimulation. Similarly, RNA obtained from the pancreatic carcinoma cells MIA-Paca-2 exhibited multiple transcripts after PMA stimulation because of alternative splicing of exon six with 3' untranslated sequences.7

Using CSF-1 probe with high specific radioactivity (3 × 10⁹ cpm/μg) and after prolonged exposure, we could observe only the 4-kb message in D-BLCL after irradiation. However, in the same gel and autoradiograph, the comparison with a T-lymphocyte clone (γ6, taken 6 hours after stimulation with PMA + CaI) clearly showed a stronger 4-kb signal together with additional bands and a major one around 2-kb for the cell clone. Finally, a weak signal is present in stimulated clones at day 6 to 7 following irradiated D-BLCL addition to the culture. This signal could be observed in the 11 clones tested after a prolonged autoradiography exposure and is likely to be caused by the persistence of CSF-1 transcripts for several days after activation.

Because CSF-1 activity has been already detected in concentrated supernatants of EBV-B cell lines,22 we further attempted to evaluate the amount of CSF-1 protein effectively present in the culture medium of D-BLCL-stimulated T-cell clones.

CSF-1 protein production by T-cell clones. In some initial experiments, the secretion of CSF-1 by the human ATLClcs, 2F7 and 4W, was evaluated by collecting culture supernatants at 5, 11, 24, 48, 72, and 96 hours of culture after specific-antigenic stimulation with D-BLCL and assaying for CSF activity in a day-7 murine methylcellulose colony assay. In this murine bone marrow assay human GM-CSF and IL-3 have no effect. In addition, RNA blot analysis of the same responder cells with G-CSF-specific probe did not show any signal either after PMA + CaI or after antigenic stimulation (data not shown), therefore precluding any involvement of the G-CSF protein in the growth of murine bone marrow colonies, which were exclusively found to be of the macrophage phenotype. Figure 4 shows that CSF
activity began to accumulate in the 2F7 culture supernatant by 24 hours after addition of stimulators and the level continued to increase after 96 hours. CSF activity 4W culture medium became significantly detectable 48 hours poststimulation, and further increased. These results fit well with the situation observed at the RNA level for the 4W clone, which did not clearly exhibit a CSF-1 transcript 24 hours after antigenic stimulation, suggesting delayed kinetics of activation. CSF-1 activity was readily detectable at 5 hours after PMA + CaI stimulation of both ATLCs, with no further increase until 24 hours.

CSF-1 radioimmunoassay (RIA) on 2F7 culture supernatants confirmed that the macrophage-stimulating activity detected in colony assays was indeed caused by immunologically reactive CSF-1 protein. The per day production of CSF-1 protein by 2F7 cells following stimulation with irradiated D-BLCL were serially measured by a this RIA. Results of one experiment are given in Table 2. Culture medium exhibited a background of 120 U/mL, which has been substrated from data. CSF-1 released by 2F7 cells on D-BLCL challenge reached a maximum at day 2 and thereafter decreased to a plateau level until day 7, underlining the close relationship between gene transcription and protein secretion. As a control, we assessed CSF-1 production under the same conditions by irradiated D-BLCL. Although irradiated D-BLCL produced the protein, it cannot alone account for the total release of CSF-1 in these cultures. To verify that the CSF-1 detected by radioimmunoassay was biologically active, 2F7-conditioned media were tested in a collagen murine colony assay. Concentrated supernatants of 2F7 cultures activated with irradiated D-BLCL stimulated murine progenitor cells to grow specific anti-rhu CSF-1 rabbit antiserum, which by itself had no effect on colony growth, significantly reduced the macrophage colony-forming ability of these preparations (Table 3). This observation demonstrates a major involvement of CSF-1 derived mainly from T cells in these experiments. In this set of experiments we have checked the monocytic nature of the colonies by staining for butyrate esterase.

**DISCUSSION**

In the present paper we report on the CSF-1 gene expression and the corresponding protein production by 11 T-lymphocyte clones from different origins exhibiting a large phenotypic diversity.

The biologic effects of CSF-1 are mainly restricted to the mononuclear phagocytic lineage and the specific cell receptor that mediates this effect, the protooncogene product c-fms, occur almost exclusively on cells of the same lineage. In humans, the biologically active molecule is found in urine and normal sera. It is produced by non-T-cell tumor cell lines such as MIA-Paca-2 (pancreatic carcinoma), GCT-C (metastatic fibrous histiocytoma),17 and TPA30.1 (SV40-transformed trophoblast cells). In addition, it has been recently reported that treatment of mouse bone marrow cultures with culture supernatants from ovarian cancer cells stimulated the production of macrophages colonies and that this effect was because of CSF-1 production by tumor cells. Conversely, the presence of phagocytic and adherent cells increased the cloning efficiencies of ovarian tumor cells in semisolid medium, whereas their depletion lead to a decreased cloning efficiency demonstrating complex interactions between ovarian tumor cells and normal, phagocytic, tumor-infiltrating cells.
physiologic circumstances CSF-1 has been implicated in placental development and several normal cell types, including monocytes/macrophages, fibroblasts, stromal cells, and endothelial cells produce CSF-1 either constitutively or after stimulation. Until recently, the T-cell lineage was thought to be incapable of producing CSF-1.

The concept that T cells do not produce CSF-1 has been questioned. Recent reports have shown that PMA-stimulated PBLs either produced the protein or expressed the mRNA for this factor. However, in these studies, no details were presented on the likely contamination of the T-cell population by monocytes and, therefore, no definitive conclusion could be drawn concerning the ability of T lymphocytes to produce CSF-1. Nonetheless, it is clear that some T-cell lines of leukemic origin either transcribed this gene or secrete the protein in the culture supernatant. This result has been reported for C10-MJ2 cells, an HTLV-1-positive T-cell line, following lectin stimulation and also for a clone of the lymphoblastoid T-cell line CEM-ON, which constitutively secreted CSF-1 in sufficient quantity to allow its identification through amino-terminal microsequencing. However, in this latter case it is not known whether the CSF-1 production by CEM-ON truly reflects a normal behaviour of T cells during the immune response because the factor production was not enhanced after lectin stimulation. These two pieces of data suggested that T cells produce CSF-1 that would, in addition to regulating the immune and/or inflammatory systems, regulate growth and differentiation of hematopoietic cells.

CSF-1 expression by malignant T-cell lines prompted us to test normal T-cell clones for the ability to produce this cytokine. For this purpose, we exploited alloreactive T-cell clones that we had previously generated from kidney allograft infiltrating cells (ATLCs) from allograft infiltrating cells (ATLCs) from GVHD patients or LGLD to examine the potential CSF-1 gene transcription from these T-cell clones representing a variety of conditions.

<table>
<thead>
<tr>
<th>Conditions of Stimulation</th>
<th>Normal Rabbit Serum 1:100*</th>
<th>Rabbit Anti-CSF-1 1:100*</th>
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<tbody>
<tr>
<td>Irradiated B cells: 24 h</td>
<td>33 ± 3</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Irradiated B cells: 72 h</td>
<td>27 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>rhuCSF-1 Behring (500 U/mL)</td>
<td>32 ± 5</td>
<td>3 ± 1</td>
</tr>
</tbody>
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*Murine bone marrow cells, 1.2 × 10⁶, were plated in 0.3 mL collagen culture in 24 wells containing 10% of 2F7-conditioned medium, previously concentrated 5 to 10 times on Minicon macrosolute concentrator (Amicon, Danvers, MA). Cultures were incubated with either NRS or anti-CSF-1 antiserum at a 1:100 final dilution as described for 7 days at 37°C with 5% CO₂ in air.
of distinct phenotypes. Of the 11 clones tested all contained significant amounts of CSF-1 mRNA when stimulated by PMA + Cal. The peak of CSF-1 mRNA appeared at about 5 hours after stimulation and rapidly decreased thereafter. Although this type of stimulation is not "physiological" in nature, it does, however, demonstrate that whatever the T-cell surface phenotype, there is no block of the CSF-1 gene transcription. This result is not the case for G-CSF, for example, with the same panel of T-cell clones under identical culture conditions (data not shown). For ATLCs where the molecular targets are known, the specific antigenic challenge also led to mRNA induction but with slower kinetics and the peak expression was found around 11 hours postaddition of the irradiated stimulators. Eight days after the last stimulation with lectin or antigen, the cultures expressed a low level of IL-2 receptors at their surface and ceased proliferating. In this "resting" state, the 4-kb transcript was still detectable, suggesting either a long half-life for these messenger RNAs or a constitutive transcription of the CSF-1 gene at low rate, a situation similar to that mentioned earlier for monocytes.

After both specific antigenic stimulation and PMA + Cal exposure, the transcript accumulation preceded the release of a M-CSF activity by ATLCs, as tested in the murine standard bone marrow assay. However, for one ATLC (4W) the Northern blot was not conclusive for the presence of CSF-1 transcripts 24 hours after specific allogenic stimulation. Nevertheless, CSF activity was detectable in culture supernatant at 48, 72, and 96 hours, suggesting, therefore, delayed transcription of the CSF-1 message in these cells. This colony stimulating activity was identified as immunologically and biologically active CSF-1 by a specific RIA, by the exclusive monocytic colony growth, and by its abrogation by a specific anti-CSF-1 rabbit antiserum. Recently, spontaneously outgrown Epstein Barr Virus (EBV)-cell lines and a specific anti-CSF-1 rabbit antiserum. Recently, spontaneously outgrown Epstein Barr Virus (EBV)-cell lines and normal activated T lymphocytes have been shown to produce CSF-1. We were thus concerned by the fact that the CSF-1 activity we detected in our cultures of pure T-cells stimulated with irradiated D-BLCL could have been due to release by B-cell line. Two lines of evidence argue against this hypothesis. First, the comparison between 24 hours serial analysis of the CSF-1 secretion of antigenically stimulated T-cell clones and irradiated D-BLCL. CSF-1 secretion measured by radioimmunoassay shows that irradiated D-BLCL alone produced only a low level of this factor and that the presence of 2F7 lymphocytes is predominantly responsible for the CSF-1 release observed in the supernatant of T cells after antigenic stimulation. Second, the 2F7 clone (CD8+) used in this set of experiments is highly cytotoxic against this BLCL line. It gives 30% to 40% of specific chromium release in a standard 4-hour assay at effector/target ratio of 1/1. In this context, the above mentioned results showing some CSF-1 production by irradiated D-BLCL are clearly a maximal estimates because in these experiments the cytotoxic T cells were absent. However, the kinetics of the CSF-1 release with both stimulations is different, PMA + Cal inducing a rapid transcription of CSF-1 gene. The property of T cells to transcribe and produce CSF-1 after activation may be important in vivo as well. The ability of T cells to produce CSF-1 provides yet another mechanism for lymphocytes to regulate cells of the mononuclear/phagocytic series. In this respect, it is conceivable that CSF-1 production by the activated T cells present at the site of the rejected allograft might participate in the rejection process. However, this conclusion cannot be drawn from the present experiments in the case of GVHD and LGLD because we could not test these T-cell clones for CSF-1 production stimulated with their specific antigens because they are currently unknown and, second, the cells from which these clones were derived did not bear (skin biopsies) any activation marker (IL-2 receptor [IL-2-R], Class II antigens) in vivo.

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MM Hallet, V Praloran, H Vie, MA Peyrat, G Wong, J Witek-Giannotti, JP Soulillou and JF Moreau