Synergistic Stimulation of Macrophage Proliferation by the Monokines Tumor Necrosis Factor-Alpha and Colony-Stimulating Factor 1

By Donald R. Branch, A. Robert Turner, and Larry J. Guilbert

The effects of pure recombinant human tumor necrosis factor-alpha (TNF), originally described for its cytostatic and cytoidal effects on neoplastic cells, and CSF-1, the macrophage-specific, colony-stimulating factor, both these factors have been well characterized, purified to homogeneity, and molecularly cloned. CSF-1 strongly promotes mononuclear phagocyte survival, function, proliferation, and differentiation. TNF acts beyond the original definition and has been shown to exert multifunctional effects on normal cells, including hematopoietic cells. It inhibits colony formation stimulated by CSFs, induces, in cooperation with gamma interferon, monocytic differentiation of the human myelomonocytic cell line HL-60; and stimulates production of CSF-1 from human monocytes. Conversely, CSF-1 can induce production of TNF from monocytes.

The above observations indicate that although the actions of TNF and CSF-1 exert different effects on hematopoietic cells, they may be linked via reciprocally induced production by monocytes/macrophages. Whether the two monokines also modulate each other's actions on macrophages is unknown. We have therefore asked in this report whether TNF modifies in any way the growth-stimulating effects of well-defined populations of murine macrophages are examined. Primary bone marrow–derived macrophages (BMM) from endotoxin-resistant C3H/HeJ mice were characterized for homogeneity in comparison with a cloned, growth factor–dependent macrophage cell line (S1) also derived from C3H/HeJ bone marrow cells. The mitogenic effects of each factor, alone and in combination, on the proliferation of both macrophage populations over a two-day culture period were studied. In contrast to CSF-1, TNF alone only slightly stimulated macrophage proliferation. However, the combination of CSF-1 and TNF stimulated proliferation of both primary BMM and S1 cells 1.5- to 2-fold greater than the sum of their predicted individual contributions. Such synergy was observed even at very high (plateau) levels of factors. TNF was found to transiently down-regulate CSF-1 receptor levels on both populations. Down-regulation was maximal at one hour; however, receptor numbers returned to initial, or greater, levels after 24 hours of incubation. Thus, TNF, an inducible monokine, greatly enhances the maximal mitogenic effects of CSF-1, an inducer of TNF production. These observations suggest an autocrine rule for TNF that involves synergy with (and perhaps obligatory cooperation with) CSF-1 in the regulation of macrophage proliferation.

MATERIALS AND METHODS

Cells. BMMs were prepared from the femurs of C3H/HeJ (H-2b) mice as previously described, with modification to allow harvest of adherent cells at day seven of culture. Bone marrow cells were cultured in tissue culture dishes at 10^6/mL in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) supplemented with 15% (vol/vol) fetal bovine serum (FBS, Flow Laboratories, McLean, VA), 10% (vol/vol) medium conditioned by the myelomonocytic cell line WEHI-3 (American Type Tissue Collection, Rockville, MD), and 2,000 U/mL stage 1 murine CSF-1. After 24 hours, nonadherent cells were transferred to new tissue culture dishes with fresh growth medium and cultured for an additional 12 days, at which time nonadherent cells (d3BMMs) were harvested and cryopreserved in liquid nitrogen. When required, frozen aliquots of d3BMMs were thawed and plated into culture in Petri plastic dishes with IMDM containing FBS (15% vol/vol) and stage 1 CSF-1 (3,000 U/mL). After two days of culture, the nonadherent cells were harvested and replated into new Petri plastic dishes with fresh CSF-1 (3,000 U/mL)-containing growth medium. After two additional days in culture, adherent cells (d7BMM) were harvested following five minutes of treatment at 4°C with 5 mmol/L ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS).

The growth factor–responsive murine macrophage cell line 5/10.14 was isolated from v-myc-transformed C3H/HeJ bone marrow cells in our laboratory as previously described. The S1 line was cloned from 5/10.14 by dilution culture with high concentrations of CSF-1 and GM-CSF and was selected for low autonomous growth and high responsiveness to CSF-1, granulocyte-macrophage (GM)-CSF, and IL-3. Early passage aliquots are cryopreserved and, prior to any experiment, recultured for one to three days in Petri dishes containing IMDM supplemented with 15% FBS, 20% L-cell conditioned medium, and 10-20 ng/mL recombinant GM-CSF.

Cell surface marker analysis and morphology. The rat monoclonal antibody (MoAb) F4/80 was used as unpurified culture supernatant of the F4/80 hybridoma obtained from Dr. S. Gordon. Monoclonal rat antibody to the macrophage C3bi receptor (MAC-1) was used as supernatant from the original hybridoma (clone 307).
M1/70.15.11.5, American Type Tissue Collection). Murine monoclonal anti-H-2K\(^d\) (IgG2a, clone 16-3-1N)\(^a\) and anti-H-2\(d\) (IgG2a, clone 34-5-8s)\(^b\) were used as unpurified ascites. Murine monoclonal anti-I-A\(^d\) (class II, IgG2a, clone 10-3.6.2)\(^b\) and anti-I-A\(^d\) (IgG2a, clone MK-D6)\(^b\) were used as unpurified ascites. Murine monoclonal IgM anti-Thy-1.2 was purchased from New England Nuclear, Boston. Murine monoclonal anti-Ly-2.1 (IgG2a, clone 116-13.1) and rat monoclonal anti-L3T4 (clone GK1.5) were used as ammonium sulfate purified culture supernatant from the original hybridoma (American Type Tissue Collection). Murine monoclonal IgM anti-asialo-GM\(_1\) (clone 49H8)\(^b\) was used as an affinity-purified ascite. Cell-surface antigen expression was assessed by indirect immunofluorescence using fluorescein (FITC)-conjugated affinity purified F(ab')2 fragment goat anti-mouse Ig and goat anti-rat IgG (Cappel Laboratories, Cooper Biomedical, Malvern, PA) and analyzed on a Coulter EPICS V flow cytometer. Cells were preincubated for 30 minutes at 4\(^\circ\)C with undiluted normal goat serum. The serum was removed and the cells were resuspended in diluted PBS containing 10% (vol/vol) normal human AB serum and 2% (vol/vol) FBS. An equal volume of MoAb was added to cells, incubated for one hour at 4\(^\circ\)C, and washed twice with PBS. One hundred microliters of the appropriate FITC-conjugated second antibodies were added for another hour's incubation at 4\(^\circ\). The cells were washed twice with PBS and resuspended in PBS containing 1% (vol/vol) formaldehyde prior to fluorescence analysis. Background immunofluorescence was determined using PBS. Negative and isotype controls were compared with background levels. Phagocytosis was assessed by uptake of FITC-impregnated latex beads (Polysciences, Warrington, PA) after incubation for one hour at 37\(^\circ\)C. Cytospin preparation of cells from log-phase growing cultures were prepared and stained with Wright-Giemsa before differential analysis. A minimum of 200 cells were counted and observed for characteristic morphologic features.

**Cytokines.** Pure recombinant human TNF (10\(^8\) U/mg) was purchased from Amgen Biologics, Thousand Oaks, CA stored at 2-8\(^\circ\)C, and used within two months of arrival. Highly purified, murine CSF-I\(^b\) was a gift from Dr E.R. Stanley, New York.

**Proliferation assays.** Proliferation of macrophages in two day cultures were monitored by uptake of radioactive thymidine (\(^3\)H-TdR, New England Nuclear, Boston, 20 Ci/mmol, 1 \(\mu\)Ci/microwell) in the last four hours of culture using a previously described 96-well microtiter plate method.\(^{44}\) Cytokines were diluted in IMDM containing 15% (vol/vol) FBS to give a final volume of 50 \(\mu\)L/well. Cells for proliferation assays were harvested from Petri dishes as described above and washed four times with sterile PBS, and 10\(^4\) cells in 50 \(\mu\)L were added to all wells of the microtiter plate. The plates were incubated at 37\(^\circ\)C in an atmosphere of 5% CO\(_2\) in air for two days, and then \(^3\)H-TdR was added for four hours. After the strongly adherent macrophages were loosened with 0.05% Triton X-100 for five to ten minutes, the cells were harvested using a Titerek 550 cell harvester (Flow Laboratories), and \(^3\)H-TdR incorporation was measured on a scintillation counter.

\(^{125}\)I-CSF-I binding assays. CSF-I was radioiodinated with Chloramine T as previously described to approximately 3 x 10\(^8\) cpm/moll.\(^{12}\) The biologic activity of each iodinated preparation was determined using a previously described colorimetric (MTT) assay.\(^{12}\) Binding of \(^{125}\)I-CSF-I was performed at 4\(^\circ\)C as previously described,\(^{12}\) with modification to accommodate analysis in 96-well microtiter dishes. Log-phase growing cells were harvested and washed four times with PBS, resuspended in IMDM containing 15% (vol/vol) FBS, and aliquoted at 2 x 10\(^4\) per well per 50 \(\mu\)L. After overnight incubation in the absence of CSF-1 to up-regulate CSF-1 receptors,\(^{44}\) TNF was added to selected wells at a final concentration of 1,600 ng/mL for one, two, four, and 24 hours. Each plate was washed twice with 200 \(\mu\)L well ice-cold PBS; 50 \(\mu\)L of 1MB (IMDM lacking bicarbonate and adjusted to 280 mosm and pH 7.35)\(^{14}\) containing 10% FCS (vol/vol) was added to each well; and the plate was cooled on ice for 20 minutes. After cooling, 10 \(\mu\)L of \(^{125}\)I-CSF-1 (50,000 cpm) were added to each well, the solutions quickly mixed on a shaker, and the reaction allowed to proceed for two hours on ice. Nonspecific binding was determined by preincubinating the cells for two hours with a saturating level (2 nmol/L) of nonradioactive CSF-I before addition of \(^{125}\)I-CSF-I.\(^{13}\) The binding reaction was stopped by washing six times with 200 \(\mu\)L ice-cold PBS. Cells-associated \(^{125}\)I and DNA were quantitatively recovered from individual microwells following a 30-minute incubation with 100 \(\mu\)L 0.1 mol/L NaOH at 60\(^\circ\)C. The contents of each well were combined with washes of 100 \(\mu\)L of 0.1 mol/L tris (hydroxymethyl) aminomethane containing 0.1 N HCl (final pH 8.0) and 100 \(\mu\)L of 5 mol/L NaCl. Samples were counted for \(^{125}\)I on a gamma counter, and then the DNA content of individual samples was quantitated fluorometrically on a TKO 100 mini-fluorometer (Hoefer Scientific, San Francisco) after reaction with bis-benzimidazole (Hoechst 33258, Sigma Chemical Co, St Louis).\(^{24}\) The DNA content of all microwells containing either BMM or S1 cells was the same (36 ± 3 ng per 2 x 10\(^4\) cells); thus, the binding results are given in terms of cpm per 2 x 10\(^4\) cells added.

**RESULTS**

**Characterization of macrophage populations.** The primary d7BMM and the S1 cell line were comparatively characterized for homogeneity, morphology, and expression of cell-surface markers that typify macrophages (Table 1). Both populations are adherent and phagocytic to the same degree and express similar levels of cell-surface markers indicative of relatively mature macrophages (eg, F4/80, MAC-1, class II). Morphologically, both populations also show similar features (eg, single nucleus, intermediate nucleus-to-cytoplasm ratio, slightly basophilic and vacuolated cytoplasm) typical of monocytes/mature macrophages. Therefore, within the resolution of this analysis, primary d7BMM are almost indistinguishable from the cloned S1 macrophage cell line.

**Stimulation of macrophage proliferation by TNF and CSF-I.** CSF-I, when added at maximally stimulating (pla-
teau) or at suboptimal levels, stimulates the proliferation of both macrophage populations (Table 2). TNF alone has almost no effect on the proliferation of murine macrophages, although in nine independent experiments using high concentrations (≥1,250 ng/mL), 3H-TdR uptake was invariably above control levels. In contrast to its marginal effect alone, TNF more than doubles the maximal ability of CSF-1 to stimulate d7BMM proliferation (Table 2). Indeed, at levels of less than 200 ng/mL, TNF does not significantly stimulate macrophage proliferation but is still able to strongly enhance (>twofold) CSF-1 stimulation of proliferation. The same results (synergy between TNF and CSF-1) were obtained in eight independent experiments using 3H-TdR uptake as an index of growth and in yet another experiment in which MTT reduction was used as a measure of viable cell number (data not shown). The fact that this action of TNF is not mediated by a non-macrophage accessory cell in the primary population is shown by almost identical effects on the S1 cell line.

Effect of TNF on binding of CSF-1 to macrophages. Comparison of binding assays performed under the same conditions (125I-CSF-1 concentration, cell number, temperature, and time of reaction) shows S1 cells to have approximately one third the number of specific CSF-1 binding sites that d7BMM have (Table 3). Incubation of both populations with TNF at 37°C caused rapid and almost complete (87%-94%) loss of CSF-1 binding. The down-modulation of binding sites by TNF was transient, with specific binding reaching its minimum at one hour and returning to normal (d7BMM or greater [S1]) values by 24 hours (Table 3). This transience was not due to loss of TNF in culture, because significant levels of TNF remain even after three days of culture (data not shown; determined by lysis assay using TNF-sensitive murine L-929 cells).

DISCUSSION

In contrast to previous reports of antiproliferative effects of TNF on immature hematopoietic cells, this report documents a novel, growth-stimulating action on more mature macrophages. TNF alone appears to be marginally mitogenic. However, this effect could well be due to its induction of CSF-1 release, which occurs with both primary d7BMM and the S1 macrophage cell line (data not shown), and which others have reported with human monocytes. The major stimulatory effect of TNF is in cooperation with CSF-1, hitherto regarded as the most potent stimulator of murine macrophage proliferation. The synergistic stimulation of growth by CSF-1 and TNF is observed with primary cells and a cloned cell line, both of C3H/HeJ origin; thus, the effects of TNF are not mediated by bacterial lipopolysaccharide (LPS) contaminants, are exerted on the same cells that bear the CSF-1 receptor, and are not limited to cell lines. Indeed, because the macrophage is the major TNF-producing cell, cooperation between TNF and CSF-1, the macrophage-specific growth factor, would most likely occur in vivo.

TNF may enhance the mitogenic action of CSF-1 by mimicking, at greater amplitude, a part of the usual pleiotropic response to the growth factor. One action that TNF mimics at greater amplitude is down-modulation of CSF-1 binding (Table 3). CSF-1 only partially down-regulates its own receptor on d7BMM, whereas TNF rapidly and almost completely does so. This down-modulation of CSF-1 binding by TNF is not due to receptor cross-reactivity, because TNF

### Table 2. Stimulation of Macrophage Proliferation by CSF-1 and TNF Alone and in Combination

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Maximum Levels†</th>
<th>Suboptimal Levels‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated Additive</td>
<td>Observed</td>
</tr>
<tr>
<td>d7BMM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>396 ± 132</td>
<td>337 ± 52</td>
</tr>
<tr>
<td>TNF</td>
<td>591 ± 135</td>
<td>300 ± 145</td>
</tr>
<tr>
<td>CSF-1</td>
<td>4,852 ± 344</td>
<td>3,134 ± 257</td>
</tr>
<tr>
<td>TNF + CSF-1</td>
<td>5,443 ± 479</td>
<td>3,434 ± 402</td>
</tr>
<tr>
<td>S1 Cell Line</td>
<td>10,424 ± 386</td>
<td>8,069 ± 673</td>
</tr>
</tbody>
</table>

*Cpm ± SD of 3H-TdR uptake.
† Maximum stimulatory (plateau) levels when used alone: TNF = 1,250 ng/mL; CSF-1 = 2.5 × 10^4 U/mL.
‡ Suboptimal stimulatory levels: TNF = 125 ng/mL; CSF-1 = 25 U/mL (d7BMM) and 100 U/mL (S1 cell line).
§ ∑ means ± ∑ SD.
|          |                   |                 |
| d7BMM    | 26,040 ± 1,871    | 22,363 ± 449    |
| S1 Cell Line | 41,973 ± 2,809  | 7,885 ± 620     |

### Table 3. Effect of TNF on 125I-CSF-1 Binding at 4°C

<table>
<thead>
<tr>
<th>Conditions</th>
<th>d7BMM</th>
<th>S1 Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-specific binding</td>
<td>67 ± 10</td>
<td>71 ± 12</td>
</tr>
<tr>
<td>Medium alone</td>
<td>3,168 ± 269</td>
<td>1,149 ± 139</td>
</tr>
<tr>
<td>TNF for one hour</td>
<td>492 ± 43</td>
<td>178 ± 25</td>
</tr>
<tr>
<td>TNF for two hours</td>
<td>1,178 ± 45</td>
<td>365 ± 37</td>
</tr>
<tr>
<td>TNF for four hours</td>
<td>2,195 ± 212</td>
<td>746 ± 18</td>
</tr>
<tr>
<td>TNF for 24 hours</td>
<td>3,648 ± 497</td>
<td>4,035 ± 327</td>
</tr>
</tbody>
</table>

* Cpm per 2 × 10^6 cells; assays done in duplicate.

See Materials and Methods section.
does not compete with 125I-CSF-1 for binding to its receptor at 4°C (data not shown). The correlation between transient and complete down-modulation of CSF-1 receptor and synergistic growth stimulation of macrophages has been observed before with tumor-promoting phorbol diesters,27,28 which are direct activators of protein kinase C,29 and with other hematopoietic growth factors.30,31 These results therefore indicate that receptor down-modulation is either important to the mitogenic action of CSF-1 or reflects an important response.

Alternatively, TNF may exert an effect on macrophages that CSF-1 cannot; i.e., it may complement CSF-1 to stimulate growth. Because CSF-1 has been reported to induce TNF production from mononuclear phagocytes,31 no mitogenic response to CSF-1 may be free of TNF effects. Thus, the possibility exists that the macrophage, like its progenitors,32,33 may require other growth factors in addition to CSF-1 for proliferation, and that TNF may be one of these requirements. We are presently exploring this possibility.

ACKNOWLEDGMENT

We wish to thank Dr E.R. Stanley for the gift of pure murine CSF-1, Dr M. Crainie for her gift of murine monoclonal anti-I-A,9, and Dr M. Sadelain for the generous gifts of murine monoclonal anti-H-2Kk, anti-H-2Dd, anti-I-A, anti-asialo-GM1, and rat monoclonal anti-L3T4.

REFERENCES

28. Chen B D-M, Lin H-S, Hsu S: Tumor-promoting phorbol esters inhibit the binding of colony-stimulating factor (CSF-1) to


Synergistic stimulation of macrophage proliferation by the monokines tumor necrosis factor-alpha and colony-stimulating factor 1

DR Branch, AR Turner and LJ Guilbert