Multilineage Synergistic Activity Produced by a Murine Adherent Marrow Cell Line

By Peter Quesenberry, Zengxuan Song, Elizabeth McGrath, Ian McNiece, Richard Shadduck, Abdul Wahed, Gwen Baber, Ellen Kleeman, and Donald Kaiser

We reported previously that a cell line (TC-1) derived from adherent marrow cells produced colony-stimulating factor 1 (CSF-1) and an activity that interacts synergistically with CSF-1 to stimulate giant macrophage colonies. We now report that an activity in TC-1 conditioned media (CM) separate from CSF-1 also synergizes multilineage colony formation by pure interleukin 3 (IL 3) and a crude source of granulocyte-macrophage colony-stimulating activity (GM-CSA) (murine lung-conditioned media). IL 3-induced megakaryocyte colony formation is also synergized. The CSF-1-dependent synergistic activity is not blocked by antibodies to IL 3 and is characterized as a nondialyzable (mol wt cutoff 3,000), heat-stable (56 °C, 30') activity that binds to DE-52 cellulose under conditions in which IL 3 does not.

**Materials and Methods**

**Cell Lines**

**TC-1 cell line**. The TC-1 cell line, initially derived from C57Bl/6J murine marrow, was maintained in Fischer's media supplemented with 20% fetal calf serum (FCS), penicillin, and streptomycin. The cultures were incubated at 33°C in 5% CO2 and trypsinized (0.1%) and passaged every 4 to 7 days. CM were collected, centrifuged at 1,500 g, filtered through a 0.45-tim Millipore membrane, and stored at -20°C.

**Factor-dependent cell lines**. FDC-P1 cells (courtesy of Dr Jim Ihle, Frederick, MD) were grown in RPMI 1640 (GIBCO, Grand Island, NY) with 10% FCS and 25% CM from the WEHI cell line. The cells were incubated at 37 °C and 5% CO2 in 25-cm2 Corning flasks. Flasks were inoculated with 2 x 105 cells/mL and split 1:10 twice a week. DA-1 cells (courtesy of Dr Jim Ihle) were handled in a similar fashion.

**Isolation of TC-1 CM-responsive FD cell lines**. FDC-P1 cells were cloned in a soft-agar McCoy's clonal culture system (as used for murine marrow colony-forming assays) (described later), and in the presence of 10% to 20% x TC-1 CM or 6% TC-1 CM partially purified by diethylaminoethanol (DEAE) chromatography. FDC-P1 colonies that formed after 14 and 24 days of growth were aspirated with a microliter pipette and dispersed in 35-mm Petri dishes containing 2 mL of RPMI 1640, 10% FCS, and 50% TC-1 CM. After 3 days, the cells were transferred to 60-mm Petri dishes (5 mL) and 3 days later were transferred to 25-cm2 Corning flasks at 2 x 105 cells/mL. The cells were then split 1:5 to 1:10 twice weekly with reseeding kept at 2 x 105 cells/mL. After 2 weeks, cells were maintained in RPMI, 10% FCS, with 25% TC-1 CM.

**Growth Factor Assays**

**Clonal agar assays**. Marrow cells from tibias and femurs of normal ICR (Dominion Labs, Dublin, VA) or normal BDF1 (Jackson Labs, Bar Harbor, ME) mice, aged 11 to 16 weeks, or BDF, mice given 150 mg/kg 5-fluorouracil (5-FU) intravenously (IV) 3 days prior to being killed were used as assay target cells. Alternatively, the factor-dependent cells FDC-P1 and DA-1 were used as assay cells in the same system. Growth factors were assayed using a modification of a double-layer agar technique or a single-layer technique. McCoy's media was supplemented with 0.8% minimal essential medium (MEM) essential amino acids, 0.4% MEM non-essential amino acids, 2 mmol/L of L-glutamine, 16 μg/mL of L-asparagine, 8 μg/mL of L-serine, 1 mmol/L of sodium pyruvate, 1 x 104 mol/L of 2-mercaptoethanol, and 15% pretested FCS. Marrow or factor-dependent cell line cells were included at different
concentrations directly in the 0.3% agar-McCoy's layer. The substances to be assayed for growth factor activity were included in different concentrations in the 0.5% agar-McCoy's underlayer in most experiments with the double-layer systems; however, in some experiments using either single-layer or double-layer cultures, the stimulus was included in the 0.3% agar-McCoy's layer. These soft agar cultures (35-mm Petri dishes, Linbro 24-well plates, microtiter, or Terasaki dishes) were then incubated at 37°C, 5% CO2 for varying time intervals (1 to 22 days), dependent on the particular experimental protocol. IL 3 purified from WEHI-3-conditioned medium11 was obtained from Dr. James Ihle and CSF-1 purified from L cell CM12 was obtained from Dr. Richard Shadduck. Murine lung CM, prepared from ICR mice as previously reported,13 was used as a source of GM-CSA. Alternatively, CM from Cononavalin A (Con A)-stimulated Balb-C spleen cells was separated by DEAE-cellulose and Sephadex chromatography as described by Ihle and co-workers.14 Material separated from IL 3 and stimulating GM colony formation was used as a source of partially purified GM-CSA.14 The morphology of colonies was determined by microscopic analysis of whole agar mount slide preparations stained with either benzidine hematoxylin or acetylcholinesterase-hematoxylin as previously reported.8

**Colony Criteria**

Colonies of >50 cells were counted as granulocyte, macrophage, GM, or mixed megakaryocyte7 and colonies that contained three or more acetylcholinesterase-positive cells were scored as megakaryocyte colonies. Routinely, colonies were scored in agar dishes or whole agar mount slide preparations using an Olympus inverted microscope with an ocular micrometer as 0.5 to 1, or >1, 2, or 3 mm in greatest diameter, and as compact or loose. The loose category included all other groupings, some of which had relatively small numbers of cells widely scattered over a 2- to 3-mm diameter.

Mixed colonies were defined as colonies with at least 3 separate areas of the minor cell type or a single area of more than three cells. Almost all mixed colonies had >20 cells of the minor type, and most GM colonies of >1 mm had large numbers of each cell type. Other special colony types are cited in the Results section.

**3HtdR Incorporation in Factor-Dependent Cell Lines**

The effects of factors on FDC-P1 or DA-1 3HtdR incorporation were assessed as described by Ihle and colleagues.14 The cells were washed three times with RPMI 1640 without WEHI-3 CM and resuspended in the same media at 1 x 10^6 cells/mL. Doubling dilutions of test samples were added to 12 microtiter wells at 50-µL vol; 50 µL of cell suspension was then added to each microtiter well. Plates were then incubated at 37°C, 5% CO2 for 24 to 72 hours and pulsed with 1 µCi/well of 3HtdR (specific activity 6.7 Ci/µmol) 5 hours prior to harvest. Cells were harvested on a Skatron harvester; 3HtdR incorporation was then determined by counting on a Beckman Beta-scintillation counter. Results are expressed as cpm.

**CSF-1 Studies and Immunoadsorption**

CSF-1 levels were determined by radioimmunoassay (RIA), and antiserum to CSF-1 was produced in male New Zealand white rabbits as previously reported.15 This antiserum was covalently bound to cyanogen bromide-activated Sepharose 4B. TC-1 CM (usually 10x concentrated) was applied to this column after preparatory dialysis against 0.05 mol/L of Tris, 0.15 mol/L of NaCl, and 0.3% polyethylene glycol (PEG) buffers, pH 7.5, to remove CSF-1. The run-through or unbound material was collected, the column was rinsed with 300 mL of buffer, and the CSF-1 was eluted with 25 mL of 2 mol/L of guanidine pH 4.0 in starting buffers. Guanidine was removed from the purified CSF by repeated dialfiltration in an Amicon ultrafiltration cell.

**Biochemical Characterization of CSF-1-Dependent Synergistic Activity**

**Collection starting material.** Initial studies were made using TC-1 CM with 5% FCS. With Nutridoma-SF supplement, the FCS concentration was reduced to 1%, and this material was used for some of the more recent studies. Thus far, reduction to no serum results in viable cells that do not produce synergistic activity. Material was pooled, and stored at -20°C until further manipulation.

**Concentration and protein determination.** Amicon ultrafiltration units or a spiral cartridge was used with YM 10 (10-kd mol wt cutoff) or YM 100 (100-kd mol wt cutoff) ultrafiltration membranes. No significant loss of bioactivity occurred in the concentrates. Total protein was determined by the Bradford assay.16

**Ammonium sulfate precipitation.** Ammonium sulfate precipitations were performed by addition of solid crystal ammonium sulfate (Ultrapure—Schwartz/Mann Biotech, Cambridge, MA). An initial cut of 0% to 35% (0.209 g/mL) was done by slow addition of crystal to a stirred flask of media. The sample was precipitated for 20 minutes at 4°C. The mixture was centrifuged at 10,000 rpm in an IEC B20A centrifuge in 200-mL vol for 30 minutes at 4°C. The supernatant was decanted, and a second cut of 35% to 65% (0.198 g/mL) was carried out. The precipitate from the second cut was resuspended in a minimal volume of 0.01 mol/L Tris-HCl pH 7.4, 0.02% Na azide, 0.01% PEG 6000, and dialyzed in the same buffer for 1 to 2 days at 4°C with three changes per day.

**DEAE-Cellulose chromatography.** DE-52 resin (Whatman, Clifton, NJ) was prepared, defined in a standard manner, and then equilibrated in 0.01 mol/L of Tris-HCl pH 7.4, 0.02% Na azide, 0.01% PEG 6000, and dialyzed in the same buffer for 1 to 2 days at 4°C with three changes per day. A Sepharose chromatography. A Sepharose S-300 column (2.5 cm x 49.5 cm, 243-mL bed volume) was equilibrated in 0.5 mol/L of NaCl, 0.02 mol/L of Tris-HCl pH 7.4, 0.02% Na azide, and 0.01% PEG 6000. Fractions of 6 mL were collected at 70 mL/h. Samples to be applied were dialyzed in the equilibrating buffer. For bioassays, fractions were pooled in groups of five and concentrated on Amicon 10K membrane threefold.

**Sephadex G100 chromatography.** Sephadex G100 (Superfine, Pharmacia, Piscataway, NJ) (2.5 x 85 cm/resin 417-mL vol) was equilibrated with 1 mol/L of NaCl in 0.01 mol/L of Tris-HCl pH 7.4 or with a linear gradient of 0 to 0.8 mol/L of NaCl. The eluate was concentrated using an Amicon spiral cartridge and 10K membrane to ~100 mL, and this concentrate was dialyzed against 0.01 mol/L of Tris HCl pH 7.4 for 1 to 2 days with three changes per day.

**Sephadex S50 chromatography.** A Sephacryl S-300 column (2.5 cm x 49.5 cm, 243-mL bed volume) was equilibrated to 0.5 mol/L of NaCl, 0.02 mol/L of Tris-HCl pH 7.4, 0.02% Na azide, and 0.01% PEG 6000. Fractions of 6 mL were collected at 70 mL/h. Samples to be applied were dialyzed in the equilibrating buffer. For bioassays, fractions were pooled in groups of five and concentrated on Amicon 10K membrane threefold.

**Con A Sepharose chromatography.** Con A Sepharose (Pharma-
MULTILINEAGE GROWTH FACTOR

Table 1. Effect of Anti-IL-3 Antibody on TC-1 CM Stimulated Giant Colony (>1 mm) Formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Giant Colony/10^6 Cells</th>
</tr>
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<tbody>
<tr>
<td>TC-1-10x (8.3%) + McCoy's 1x (8.3%)</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>TC-1-10x (8.3%) + anti-IL 3 IgG (1 mg/ml)</td>
<td>23 ± 2.4</td>
</tr>
<tr>
<td>TC-1-10x (8.3%) + control IgG (1 mg/ml)*</td>
<td>29.3 ± 2.4</td>
</tr>
</tbody>
</table>

CM, conditioned media.

In this experiment, anti-IL 3 antibody blocked colony formation by 166 U/ml of IL 3; in other experiments, a level of 1 mg/ml was sufficient to block FDC-P1 proliferation completely. *IgG purified from normal rabbit sera by protein A chromatography (courtesy of Dr James Ihle).

Fig 1. Synergistic effect of TC-1 conditioned media (CM) on Interleukin 3 (IL 3) stimulation of post-5-fluorouracil (5-FU) marrow cells. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxynlin (magnification x1.3). Murine BDF1 marrow cells were harvested 3 days after 150 mg 5-FU/kg body weight and 0.5 x 10^6 cells/ml were cultured in a double-layer agar assay with 1.7 ng/ml of IL 3 (panel A), 8.3% 20x TC-1 CM (panel B) or 1.7 ng/ml IL 3 plus 8.3% 20x TC-1 CM (panel C). Slide preparations were made after 22 days of culture.

RESULTS

Previously, we reported that the murine marrow adherent cell line TC-1 produced CSF-1 and a factor that interacted synergistically with CSF-1 to stimulate giant macrophage colonies. This factor had no intrinsic colony-forming ability, but may still represent relatively low levels of IL 3. Accordingly, we evaluated the capacity of two different antibodies to IL 3 to block giant colony formation induced by TC-1 CM. IL 3 antibodies at a concentration that abolished IL 3 colony formation and FDC-P1 (the IL 3-responsive assay cell line) proliferation did not block giant macrophage colony formation (Table 1). These data suggest that the TC-1 synergistic activity is not IL 3.

Synergism With IL 3

IL 3 has been shown to stimulate granulocyte, macrophage, GM-megakaryocyte and mixed megakaryocyte-GM colony formation. We previously established half-maximal values for IL 3-stimulated hemopoietic colony formation; depending on colony type, these ranged from 0.14 to 0.76 ng/ml. Plateau levels for all types of colony formation were achieved with concentrations of >3 ng/ml and, using these plateau levels of IL 3, we previously showed that crude TC-1 CM (with CSF-1) interacts with IL 3 to induce giant mixed GM and GM-megakaryocyte colonies from normal murine marrow. In this setting, total colony formation is increased only modestly by the combination of TC-1 CM and IL 3, with most of the increase accounted for by GM colonies (Table 2). The major synergistic effect is thus on the size and cell content rather than on the number of colonies. Furthermore, only with the IL 3/TC-1 CM combination were giant compact colonies of granulocytes, macrophages, and megakaryocytes seen. In the present studies, we show a similar potentiating effect of TC-1 CM plus IL 3 on target marrow harvested at 1 and 3 days after 5-FU 150 mg/kg, but peak growth and maximum effect occurred somewhat later than with normal marrow, ranging from 14 to 24 days of growth in soft agar culture (Fig 1). Potentiation of IL 3-induced colony formation by TC-1 CM occurred at from 0.52 to 16.7 ng IL 3/ml and was noted at different TC-1 CM plateau levels (data not shown). Because CSF-1 acts synergistically with IL 3 to give rise to giant macrophage colonies and CSF-1 is present in the crude TC-1 CM, it was important to study the role CSF-1 might play in the observed synergism between TC-1 CM and IL 3. Accordingly, we studied TC-1 CM that had been totally depleted of CSF-1 (as determined by RIA) by affinity CSF-1 antibody column chromatography. Three different pools of TC-1 CM (concentrated ten times) were passed over a Sepharose column to which

Table 2. Colony Formation (>50 Cells) Stimulated by Different Hematopoietic Growth Factors

<table>
<thead>
<tr>
<th>Colony</th>
<th>TC-1</th>
<th>IL 3</th>
<th>LCM</th>
<th>TC-1 + LCM</th>
<th>TC-1 + IL 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro</td>
<td>109 ± 10</td>
<td>72 ± 9.7</td>
<td>28.7 ± 4.6</td>
<td>33.1 ± 4.7</td>
<td>92.3 ± 11.5</td>
</tr>
<tr>
<td>GM</td>
<td>26.1 ± 4.7</td>
<td>26.5 ± 6.8</td>
<td>50 ± 5.6</td>
<td>71.1 ± 5.3</td>
<td>57.7 ± 4.2</td>
</tr>
<tr>
<td>Gran</td>
<td>16.4 ± 3.2</td>
<td>31.8 ± 7.9</td>
<td>25 ± 6.5</td>
<td>20.3 ± 1.6</td>
<td>24.2 ± 3</td>
</tr>
<tr>
<td>Mega</td>
<td>0</td>
<td>8.8 ± 3</td>
<td>2 ± 0.6</td>
<td>1.25 ± 0.8</td>
<td>5.9 ± 2.3</td>
</tr>
<tr>
<td>Total</td>
<td>151 ± 11.4</td>
<td>139 ± 10.2</td>
<td>106 ± 6.8</td>
<td>126 ± 7.8</td>
<td>180 ± 9.8</td>
</tr>
</tbody>
</table>

IL 3, Interleukin 3; Macro, macrophage; GM, granulocyte-macrophage; Gran, granulocyte; Mega, all megakaryocyte colonies of over 3 cells plus mixed megakaryocyte colonies; total, sum of all colony types.

Data are presented as mean ± 1 SEM from 8 to 9 individual slide preparations from 3 separate experiments. Stimuli either by themselves or in combination were tested at the following concentrations: TC-1 conditioned media (CM) 8.3% to 15.4%; IL 3 7.7 to 16.7 ng/ml; and LCM 7.7% to 8.3%.
purified anti-CSF-1 was covalently bound. The run-through or unbound fraction had no CSF-1 by RIA and did not stimulate murine marrow colony formation. The CSF-1 eluted off the column with guanidine, synergized with the unbound material to give giant colonies, confirming our previous observations. This unbound material when added at different concentrations to different levels of IL 3 (five separate experiments) potentiated megakaryocyte colony formation and the formation of giant GM and GM-megakaryocyte colonies (Table 3). The actual numbers of differentiated-sized groups fail to convey the true extent of this synergy, as illustrated in two experiments assessing synergy with normal or post–5-FU marrow (Figs 2 and 3). Colonies characteristic of IL 3 plus TC-1 stimulation were very large with a tight central core of cells consisting of granulocytes, macrophages, and megakaryocytes. Furthermore, with post–5-FU marrow colonies of >2 to 3 mm in diameter with central dense cells of >1 mm were seen only in the TC-1 unbound material + IL 3 group (7 ± 1/5 x 10⁶ cells), not in the IL 3 group.

The crude TC-1 CM plus IL 3 tested against post–5-FU marrow over 14 to 22 days of clonal agar culture had variable effects on the numbers of colonies >50 cells. The addition of TC-1 CM to IL 3 resulted in total colony formation that ranged from 56% to 95% of the combined IL 3 and TC-1 CM controls (ie, adding numbers of colonies stimulated by IL 3 alone and TC-1 CM alone). When the “unbound” TC-1 material was added to IL 3 and tested against post–5-FU marrow, total colony (>50 cells) formation was reduced to 74% to 85% of the IL 3 control. Thus, the major effect of the synergistic activity in TC-1 CM when added to IL 3 is to increase colony size with no overall stimulation (and in some cases, apparent inhibition) of total colony formation.

Similar results occurred when plateau concentrations of murine lung CM (a source of GM-CSA-2) were assayed in the presence of plateau concentrations of TC-1 CM; giant mixed GM colonies predominantly of the loose type were seen (Fig 4). Again, within a specific size range and category, colonies from the TC-1 + LCM group had more cells. Total colony formation was modestly depressed by the TC-1 + LCM combination as compared with TC-1 alone, but individual GM colony formation was augmented (Table 2). Again this synergizing effect was seen over varying concentrations of 10 x TC-1 CM (0.4% to 11.5%), LCM (0.4% to 11.5%), or 10 x LCM (1.19% to 3.8%) and when post–5-FU marrow was used as a target marrow (Fig 5). In the latter case, as with the IL 3 studies, peak growth and potentiation effect with the 5-FU marrow occurred at 14 to 22 days of soft agar culture. The effect of TC-1 CM on LCM-induced colony formation was confirmed in six separate experiments.

Table 3. Effect of TC-1 “Unbound” Fraction on IL-3-Induced Colony Formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Mega Total</th>
<th>&gt;2 mm</th>
<th>&gt;1 mm</th>
</tr>
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<tbody>
<tr>
<td>IL 3</td>
<td>7.5 ± 4.3</td>
<td>10 ± 4</td>
<td>3.7 ± 1.2</td>
</tr>
<tr>
<td>IL 3 + unbound fraction</td>
<td>17.3 ± 4.7</td>
<td>24.3 ± 6.2</td>
<td>19 ± 9.3</td>
</tr>
</tbody>
</table>

These data are from three separate experiments testing TC-1 conditioned media (CM) that had been passed over an anti-CSF-1 Sepharose column (unbound fraction) and was free of CSF-1 activity by radioimmunoassay. In these experiments, both normal murine ICR and BDF1, target marrow cells were used, and interleukin 3 (IL 3) levels varied between 43 and 160 U/mL. Unbound activity represented the run-through fraction and thus was equivalent to a 10 x TC-1 CM concentrate devoid of CSF-1. Potentiation occurred in different experiments with final unbound concentrations of 1% to 25%. Unbound fractions at higher concentrations ordinarily caused inhibition of growth but this inhibition varied with different pools, and the concentration at which it was seen varied considerably between the different pools.

Mega, pure megakaryocyte colonies of more than three cells; total mega, pure and mixed mega colonies; >2 mm mixed, colonies >2 mm in diameter predominantly granulocyte-macrophage (GM) or macrophage, but also including GM-Mega.

Fig 2. Synergistic effect of TC-1 “unbound” fraction on interleukin 3 (IL 3) stimulation of post-5-fluorouracil (5-FU) marrow cells. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxylin. Murine BDF1 marrow cells (0.5 x 10⁶/mL) were cultured in a double-layer agar assay with 2% unbound fraction (panel A), 2% unbound fraction plus 4.3 ng/mL of IL-3 (panel B), or 4.3 ng/mL of IL 3 (panel C). Slide preparations were made after 22 days of culture.

Fig 3. Synergistic effect of TC-1 “unbound” fraction on interleukin 3 (IL 3) stimulation of post-5-fluorouracil (5-FU) marrow cells. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxylin. Murine BDF1 marrow cells were harvested 3 days after 150 mg of 5-FU/kg body weight and 0.5 x 10⁶ cells were cultured in a double-layer agar assay with 2% unbound fraction (panel A), 2% unbound fraction plus 4.3 ng/mL of IL-3 (panel B), or 4.3 ng/mL of IL 3 (panel C). Slide preparations were made after 22 days of culture.

Fig 4. Synergistic effect of TC-1 conditioned media (CM) on LCM-stimulated colony formation. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxylin (magnification ×1.3). Murine BDF1, marrow cells (0.5 x 10⁶ cells/mL) were cultured in a double-layer agar assay with 11.5% 10 x TC-1 CM (panel A), 3.8% LCM (panel B), or 11.5% 10 x TC-1 CM plus 3.8% LCM (panel C). Slide preparations were made after 14 days of culture.
Characterization of the CSF-1–Dependent Synergistic Activity Present in TC-1 CM

Using giant macrophage colony formation (>0.5- and >1.0-mm diameter dense colonies) and carrying out clonal agar cultures with and without added pure CSF-1 (400 to 800 U/mL), we characterized the CSF-1–dependent synergistic activity present in TC-1 CM as nondialyzable (membrane cutoff 3,000), relatively heat stable (56°C for 30 minutes) with a pH range between 4 and 8. It is stable on storage at 4°C for at least 6 weeks and is stable to three or four freeze-thaws. During purification, synergistic activity was seen in fractions that also had significant levels of CSF-1, and adding CSF-1 in the assays did not reveal fractions with SA separate from CSF-1. Thus, the data presented represent the capacity of the various separated fractions to stimulate giant colony formation without added CSF-1. The SA was retained on both Amicon PM 10 (10,000 mol-wt cutoff) and Amicon PM 100 (100,000 mol-wt cutoff) and precipitated between 35% and 65% ammonium sulfate. The synergistic activity bound to DE-52 cellulose (Whatman, Clifton, NJ) that had been equilibrated in 0.02 mol/L of Tris-HCl, pH 7.4 (conditions in which IL 3 does not bind) and eluted from the column between 0.1 and 0.4 to 0.5 mol/L of NaCl (Fig 7). TC-1 CM after ammonium sulfate precipitation or DE-52 cellulose batch elution and separation by Sephadex G100 or Sephacryl S300 chromatography had an apparent mol wt of >60,000 (~200,000 by Sephadex G100) (Fig 8). The bulk of SA from crude or partially purified TC-1 CM bound to Con A eluting with α-methyl mannoside (Fig 9). A series of sequential separations resulted in significant purification and increase in specific activity (Table 4).

TC-1 CM was separated by DE-52 cellulose chromatography followed by G100 Sephadex chromatography. Active pools from the G100 column were rendered CSF-1–free by passage over an affinity CSF-1 antibody column. This “unbound” material synergized with stage-1 CSF-1 to give tight colonies >0.5 mm in diameter (Fig 10). The same unbound material tested at 13% concentration against normal murine marrow in the presence of IL 3 increased large (>1 mm) colony formation to 169% of the IL 3 control and, at a 5% concentration, caused a 3.14-fold increase in murine pre-B cell generation (as determined by cytoplasmic IgM-positive cells) in a liquid culture system. In addition, when target bone marrow cells from Balb/c mice harvested 8 days after 150 mg of 5-FU/kg were used and these cells were cultured for 14 days at 7% O₂, this same unbound preparation tested at 13% augmented IL 3 stimulation of colonies (>0.5 mm) by 360% of control. Similarly, material that had been subjected to sequential separation over DE-52 cellulose, G100 Sephadex and Con A chromatography (the α-methyl mannoside eluate) and that had been rendered free of CSF-1 by CSF-1 antibody affinity chromatography increased pre-B cell generation fivefold.

Other Growth-Promoting Activities in TC-1 CM

We recently reported the presence of a murine pre-B cell-inducing activity in crude and partially purified TC-1 CM separate from CSF-1. This activity was found in synergistic activity–active fractions separated by sequential
Fig 8. Separation of TC-1 conditioned media (CM) by Sephadex G100 chromatography. Two 7-L pools of TC-1 CM (5% fetal calf serum) were separated by batch diethylaminoethanol (DEAE)-cellulose chromatography, and the active salt eluates were then equilibrated with 0.01 mol/L of Tris HCl pH 7.4, 0.02% Na azide, and 0.01% polyethylene glycol (PEG)-6000. Fifty milliliters of this eluate was applied to a Sephadex G100 (2.5 x 85 cm/resin volume = 417 mL) column that had been equilibrated with 2 L of 0.01 mol/L of Tris HCl pH 7.4, 0.02% Na azide, and 0.01% PEG-6000. Fractions were collected at a rate of 60 mL/h with 16 mL per fraction, and aliquots from three fractions were combined for each pool, concentrated (1.8 to 3 times), and sterile filtered (0.22 U/mL). CSF-1 on the pools was determined by radioimmunoassay (RIA), and protein was determined by Bradford assay. Growth factor results are expressed as the number of tight colonies of >0.5-mm diameter stimulated per milliliter of CM.

DE-52 cellulose, Sephadex G100, Con A, and CSF-1 antibody affinity chromatography. We also noted an activity in TC-1 CM stimulating FDC-P1 (Fig 11) or DA-1 cell proliferation. This material also stimulated FDC-P1 cells that had been selected for TC-1 growth factor sensitivity, although the kinetics of stimulation were altered; moreover, in this case, by 74 hours of growth, there was a higher rate of proliferation of the FDC-P1 TC-1 sensitive cells grown in TC-1 CM as compared to WEHI-3 CM (a source of IL 3) (Fig 11).

DISCUSSION

We present a characterization of a multilineage synergistic activity produced by the murine adherent marrow cell line TC-1. We have shown that a non-IL 3 activity is capable of synergizing with IL 3 to produce giant mixed GM and GM-megakaryocyte colonies and to enhance megakaryocyte colony formation. These data show that CM from the cell line devoid of CSF-1, and with no intrinsic colony-forming ability by itself, contains this synergizing activity for IL 3. This synergy occurs against both normal bone marrow and marrow from animals pretreated with 5-FU (Figs 2 and 3). Similarly, an activity present in the TC-1 CM is capable of synergizing with a source of GM-CSA, i.e., murine lung CM, to give rise to giant GM colonies (Figs 4 through 6). This effect, while lessened with the removal of CSF-1, is still present. We previously showed the existence of an activity that synergizes with CSF-1; thus, the TC-1 cell line is capable of producing a bioactivity that has no intrinsic CSA but that appears to be able to augment the effects of three separate regulators—CSF-1, IL 3, and probably GM-CSA. In these same preparations, we also found an activity capable of inducing pre-B cells in short-term cultures of murine marrow cells. Thus, we found activities from the TC-1 stroma line that appear to act early in both B lymphopoiesis and myelopoiesis.

Pre-B lymphocyte-inducing factor was first found in the urine of patients with cyclical hematopoiesis. In this condition, at a time when myelopoiesis is depressed and just preceding a wave of pre-B cells in the bone marrow, high levels of a pre-B-inducing activity exists in the urine of these patients. We may be dealing with a similar type of activity from the adherent cell line described in this article. This cell line was isolated from long-term murine cultures by differential trypsinization using Fischer's media supplemented with FCS and no hydrocortisone. This was initially done to facilitate isolation but we now realize that these conditions may well be permissive for B lymphopoiesis in vitro and approach those described by Whitlock-Witte for long-term B lymphocyte cultures. In addition, Dorshkind recently showed that the classical myeloid Dexter murine cultures can be switched to cultures supporting B lymphopoiesis by altering the culture conditions. These alterations included using horse serum rather than FCS and omitting hydrocortisone. This suggests that one primary stromal cell may be able to provide the environment for supporting both B lymphopoiesis
MULTILINEAGE GROWTH FACTOR

and myelopoiesis and that the lineage favored may be dependent on the specific conditions of the cell. It is possible that the myeloid synergizing activity and the pre-B factor seen in the TC-1 supernatants represent the same factor (thus far, they co-purify in biochemical purifications) and that this factor is produced by stromal cells under either condition. The final pathway may be determined by the production of additional factors specific for B lymphopoiesis or myelopoiesis. In support of this hypothesis, the Dexter culture system appears to produce both CSF-1 and GM-CSA, thus providing the terminal differentiating hormones for myelopoiesis and that the lineage favored may be determined by the production of additional factors specific for B lymphopoiesis.

Table 4. Purification of TC-1 Derived LHGF

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tight Colony/5 x 10⁴ Cells/mg Protein</th>
<th>Total Units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;0.5 cm</td>
<td>&gt;1 cm</td>
</tr>
<tr>
<td>Original TC-1 CM*</td>
<td>59</td>
<td>31</td>
</tr>
<tr>
<td>DEAE-salt eluate</td>
<td>3,487</td>
<td>1,601</td>
</tr>
<tr>
<td>G-100 Sephadex Pool 3</td>
<td>7,996</td>
<td>3,553</td>
</tr>
</tbody>
</table>

Abbreviation: LHGF, lymphohemopoietic growth factor.

*Starting pool of 7 L of 5% TC-1. Only 50% of diethylaminoethanol (DEAE)-salt eluate went to G-100 column. G-100 active pools from a similar separative sequence were separated by Concanavalin A chromatography and eluted with a-methylmannoside. Fractions from this separation had synergizing activity but no detectable protein by Bradford assay. 15

Witte B lymphocyte cultures. Further work should clarify these alternatives.

We began a biochemical characterization of the synergizing activity using CSF-1 synergy as an assay. We found that the CSF-1 synergistic activity is nondialyzable and relatively heat stable, precipitates between 35% and 65% ammonium sulfate, and binds to DEAE cellulose under conditions in which IL 3 does not.14 Furthermore, the synergistic activity concentrates on Amicon PM100 membranes and is found near the void volume with both Sephacryl and Sephadex chromatography indicating a relatively high mol wt or, alternatively, the possibility of aggregate formation. This activity on chromatographic separations shows some polydispersity, and the bulk of the material appears to bind to Con A columns, eluting off with a-methylmannoside, indicating the presence of a glycoprotein although some of the material does not bind. Sequential separations through DE-52 cellulose chromatography and G-100 Sephadex chromatography result in a 135-fold purification, and separation of this material or similar material on Con A columns results in
fractions with no detectable protein but with significant activity. Thus, the synergistic bioactivity appears to be a relatively large mol wt glycoprotein but, given the history of other hemopoietic glycoprotein hormones, the glycoprotein may well have a much smaller mol wt with further purification. Thus far in preliminary studies, the pre-B activity has been found in active fractions through the Con A separation step. The BCGF-2-like activity has also been found in active fractions but appears to partially separate with various chromatographic steps.

Finally, we also found an activity in the TC-1 CM and in partially purified fractions that stimulates the FDC-P1 and DA-1 factor-dependent cell lines.\textsuperscript{25,28} These data probably extend further the number of activities that can stimulate these factor-dependent lines. Hapel and colleagues\textsuperscript{29} showed that these lines respond to multiple different activities, but they may provide a worthwhile tool for studies of the nature of the growth action and for a possible assay for its purification.

The TC-1-derived activity is similar to activities described previously\textsuperscript{27,28} in that it synergizes with CSF-1. These activities probably are represented by IL 3 and hemopoietin-1.\textsuperscript{3,5-28} The multilineage synergistic activity described from the TC-1 cell line does not appear to be IL 3, based on its lack of intrinsic colony-forming ability, a failure to be inactivated by an antibody to IL 3, and its behavior on DE-52 cellulose columns. It differs from hemopoietin-1\textsuperscript{4,29} in its apparent mol wt, lack of stimulation of total colony numbers in the presence of IL 3, and binding to Con A columns. The X factor described by Iscove\textsuperscript{30} using a different system of synergy may well be similar to this activity. Recently, two separate activities predominantly stimulating B cell types were described, and both were termed IL 4.\textsuperscript{31,32} Different assays were used to define these factors but a comparison of both IL 4s to our activity will clearly be appropriate.

Finally, based on the above findings and considerations, we speculate that the long-term myeloid and lymphoid in vitro cultures are controlled by a series of growth factors. One unifying multilineage synergizing activity may exist for both B lymphopoiesis and myelopoiesis that determines responsiveness of cells to terminal differentiating hormones. We propose that the pre-B-inducing activity and the myeloid synergizing activities represent this activity. Furthermore, we suggest that in the myeloid Dexter cultures the production of CSF-1 by a particular stromal cell type with a secondary production of GM-CSA by stimulated macrophages determines the final lineages seen in this system; macrophages, granulocytes, and megakaryocytes. Similarly, in the Whitlock and Witte B lymphocyte culture system, we suggest that the production of factors acting on the terminal end of the B cell differentiation pathway modulates the production of B cells. The particular culture conditions used may well determine whether these terminal differentiating hormones are produced, thus providing a possible explanation of the switching from B lymphopoiesis to myelopoiesis in these cultures when conditions are altered.

REFERENCES

18. Landreth KS, Witte P, Quesenberry PJ: A bone marrow stromal cell line produces growth factors which potentiate the generation of pre-B cells and synergize with known hematopoietic regulatory molecules. (submitted for publication)
22. Dorshkind K: Presented at Opportunities Workshop in Cell Biology: Pre-B Cell Growth Factor Conference, University of Virginia, September 20–21, 1985
Multilineage synergistic activity produced by a murine adherent marrow cell line

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