The Fractionation, Characterization, and Subcellular Localization of Colony-Stimulating Activities Released by the Human Monocyte-Like Cell Line, GCT


GCT, a human monocyte-like cell line, has been shown to release biochemically distinct colony-stimulating activities (CSAs) for mouse and human marrows. These appear to be periodate-sensitive proteins with critical disulfide bonds. One, of molecular weight 145,000 daltons, stimulates mouse macrophagic colony growth and is related to a 30,000-dalton molecule that also stimulates mouse growth. A 30,000-dalton CSA for human marrow can be separated from the molecular weight 145,000 daltons, stimulates mouse macrophagic colony growth and is related to a 30,000-dalton CSA for mouse cells (M-CSA) as well. Despite intensive efforts to purify CSAs from various human sources, none has been unequivocally purified to homogeneity. Results with partially purified preparations suggest that: (1) M-CSA and H-CSA are proteins, possibly glycoproteins; (2) both are molecularly heterogeneous; and (3) M-CSA and H-CSA are structurally and functionally different. Studies of CSA derived from heterogeneous populations of leukocytes have indicated that H-CSA is an integral component of the plasma membrane.

We have found that a permanent human macrophage-like cell culture, GCT, elaborates both H-CSA and M-CSA molecules. In preliminary communication, we described the adaptation of these cells to serum-free medium and the partial purification of H-CSA and M-CSA from serum-free conditioned medium. The present article describes further the purification and biochemical properties of CSA released by GCT cells. In addition, the subcellular localization of CSA and the structural relationship of cellular and extracellular CSA are defined.

MATERIALS AND METHODS

Preparation of Conditioned Medium

GCT cells were adapted for CSA production in serum-free McCoy's 5A medium supplemented with 0.01% polyethylene glycol 6000 (PEG 6000). For preparation of radiolabeled conditioned medium (CM), GCT cells were grown to confluency in plastic roller bottles in McCoy's 5A medium supplemented with 1% fetal calf serum (FCS). The cells were washed extensively with McCoy's 5A medium without serum and incubated for 8 hr with either 150 μCi of [14C]-amino acid hydrolysate or with 5 μCi L-[3H]-fucose in 15 ml of serum-free Hank's buffered salt solution supplemented with 0.02% PEG 6000. At the end of 8 hr, 200 ml of McCoy's 5A medium supplemented with 0.01% PEG without FCS was added to each roller bottle and incubated for 4 days, at which time the medium was harvested.

Heat Treatment

Crude CM was incubated for 2 hr at 37°C, 56°C, 75°C, and 90°C and assayed.

Protein Determination

All protein determinations were made according to the method of Lowry et al.

Assay of Crude and Semipurified Conditioned Medium

Both human and mouse marrow CFU-C assays were performed in 0.8% methylcellulose and 25% FCS in McCoy's 5A medium. Normal volunteers gave informed consent for iliac crest marrow aspiration. Their participation was approved by the Committee on Human Subjects.

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Investigations Involving Human Subjects. Dilutions of CSA samples to be assayed were made with McCoy's 5A medium supplemented with 10% FCS to prevent adsorption of CSA to Millipore filtering units. Both human (10³ nonadherent cells/ml) and BDF₁ mouse supplemented with 10% FCS to prevent adsorption of CSA to samples to be assayed were made with McCoy's 5A medium with 10% FCS. Sedimenting the cells for 5 min at 300 rpm in a Shandon cytocentrifuge, and staining with Wright-Giemsa. Eosinophilic cells were distinguished with Luxol fast blue stain.

Fractionation of CSA Molecules

Buffers used for all subsequent steps of fractionation contained 0.01% PEG 6000 and 0.01% sodium azide.

Stage II CM

Stage I CM (crude CM) was concentrated 10-30-fold by 1 of 3 methods: (1) ultrafiltration at 4°C (Amicon PM10 retentate), (2) rotary vacuum evaporation at 42°C, or (3) 30%-50% ammonium sulfate precipitation. Thereafter, CM was dialyzed at 4°C against distilled deionized H₂O. The resultant precipitate was removed by centrifugation at 9000 g for 20 min and the supernate was saved (stage II CM).

Stage III CM

Stage II CM was further purified by batch calcium phosphate gel absorption at pH 6.8.²⁸-³⁰ Calcium phosphate gel (1 ml gel/2 mg protein) was added to stage II CM, stirred gently for 2 hr at 4°C, and centrifuged at 6000 g for 10 min. After removal of the supernate, the precipitate was washed with distilled H₂O, resuspended in 1/10 the original volume with 0.1 M phosphate buffer, pH 6.8, and centrifuged at 6000 g for 10 min. The supernate was saved (stage III CM).

Enzyme treatment of stage III CM. Enzymes included crystal-line ribonuclease (75 U/mg), deoxyribonuclease (2000 U/mg), pronase-P (1 U/mg), lipase (7 U/mg), phospholipase C (5 U/mg), and α-chymotrypsin (5 U/mg). N-α-tosyl phenylchloromethyl ketone (TPCK) treated trypsin (32 U/mg) and Vibrio cholerae neuraminidase (1 U/mg) were used for enzyme susceptibility studies. Stage III CM, which contained both M-CSA and H-CSA, was dialyzed against phosphate-buffered saline (PBS), pH 7.4, and incubated with each enzyme for 6 hr at 37°C. Digestions were terminated by the addition of 500 μl of 20% deionized bovine serum albumin (BSA) and by placing the incubation samples on ice. Trypsin and a-chymotrypsin digests were terminated by the addition of phenylmethylsulfonyl fluoride (final concentration 5 mM), N-ethylmaleimide (NEM) for 2 hr at 23°C (final concentration 15 mM), or with DTT followed by NEM. Samples were dialyzed against distilled H₂O supplemented with 0.02% PEG 6000, filter sterilized, and assayed.

Stage IV CM

Stage III CM was dialyzed for 24 hr at 4°C against 0.03 M Tris-HCl, pH 7.4, and was applied to a DEAE cellulose column (2.5 x 75 cm) equilibrated with the same buffer. When the absorbance at 280 nm returned to baseline, a 500-ml linear gradient of NaCl was applied (0-0.25 M). Fractions were assayed and those containing M-CSA or H-CSA were pooled and concentrated by ultrafiltration (PM 10) to 5.0 ml.

Stage V CM

Stage IV CM was applied to a Sephacryl S-200 column (2.5 x 100 cm) equilibrated in 0.03 M Tris-HCL, pH 7.4, and eluted at a flow rate of 1 ml/min. Ten-milliliter effluent fractions containing either M-CSA or H-CSA were pooled, concentrated by ultrafiltration over a UM2 membrane, and dialyzed for 24 hr at 4°C against distilled water. The column had been previously calibrated using human IgG (145,000 daltons), BSA (67,000 daltons), cytochrome-C (13,000 daltons), and ¹⁴C-glucose (180 daltons) as molecular weight markers. For more accurate molecular weight estimates, stage IV CM was also applied to a 2.5 cm x 125 cm Ultrogel AcA54 column equilibrated in the same buffer and calibrated with blue dextran (~2 x 10⁶ daltons), BSA (67,000 daltons), ovalbumin (43,000 daltons), and ribonuclease (13,600 daltons).

Fractionation and characterization of stage VM. Since stage V CM was found to contain a high molecular weight mouse-active CSA (HMW-M-CSA) and low molecular weight mouse and human-active CSA (LMW-M-CSA, LMW-H-CSA), these activities were separately pooled, concentrated and further characterized according to the following four methods.

Preparative gradient polyacrylamide gel electrophoresis was performed according to Davis²⁴ and Ornstein.²³ Preparative 5%-10% polyacrylamide slab gels were made at 4°C with an ISCO gradient maker. A 3% acrylamide stacking gel was also used. The samples were diluted 1:1 with Davis sample buffer containing 20% w/v sucrose, and 3.0 ml were electrophoresed at 4°C for 18 hr at 75 V/slab gel. Gels were sliced in 3-mm sections and placed in dialysis bags with 3.0 ml of Davis running buffer. These bags were placed horizontally to the current flow, and proteins were electrophoreted in a horizontal gel apparatus for 4 hr at 250 V in Davis running buffer. The electrophoreted samples were dialyzed against PBS diluted 1:10 with 10% FCS and assayed.

Preparative isoelectric focusing of LMW stage V CM was performed in a 110-ml capacity column, stabilized in a 0%-50% sucrose gradient containing 4 M urea and 2% carrier ampholines (pH 3-10). The column was cooled to 5°C with running tap water, and 350 V was applied for 72 hr, after which 3.0-ml fractions were collected and their pH measured. They were dialyzed against PBS and AG 501-X8D ion exchange resin to insure removal of toxic ampholines. Aliquots were diluted 1:10 with 10% FCS before filtering and assaying.

Affinity chromatography of stage V CM was performed on concanavalin A Sepharose 4B (Con A-Sepharose). LMW CSA and HWM M-CSA were dialyzed against 0.2 M acetate containing 1 M NaCl and 1 mM (each) of MgCl₂, CaCl₂, and MnCl₂, and chromatographed on a Con A-Sepharose column (1.5 x 30 cm) equilibrated in the same buffer. Bound material was eluted with 0.2 M α-methyl mannoside (omn) in the same buffer.

SDS polyacrylamide gel electrophoresis of ¹⁴C-labeled stage
V CM was performed according to Laemmli.\textsuperscript{17} Radioautographs were made by placing Kodak X-Omat film over dried gels kept in a press in the dark for 2–10 wk.\textsuperscript{18}

**Inhibition of Protein Synthesis by Cycloheximide and Serum Deprivation**

GCT cells were extensively washed with McCoy's 5A without FCS, and 10\textsuperscript{6} cells were incubated with 5.0 ml of either standard McCoy's 5A medium with 10% FCS containing varying concentrations of cycloheximide or serum-free medium supplemented with 0.01% PEG 6000. On the third day the cell suspensions were pulse-labeled for 24 hr with 10 μCi \textsuperscript{3}H-leucine. On day 4, the supernate was removed, dialyzed against PBS, and assayed. A 200-μl aliquot of each dialyzed sample was added to 10.0 ml of Bray's solution and counted for 10 min in a scintillation counter to measure the total counts per minute (cpm) incorporated into released proteins.

**Preparation of Subcellular Fractions and Purified Plasma Membranes From GCT Cells**

Plasma membranes were prepared according to Segel et al.\textsuperscript{19} and Wang et al.\textsuperscript{8} Cells (~5 × 10⁶) were scraped off of roller bottles, washed 3 times in 0.9% NaCl, resuspended in 10 ml of lysis medium (1 mM NaHCO\textsubscript{3}, 0.5 mM CaCl\textsubscript{2}, pH 7.4), homogenized, diluted to 50 ml with lysis medium, and centrifuged at 500,000 g for 20 min. This procedure was repeated with the pellet. The pellet, designated nuclear-mitochondrial fraction, was saved. The supernates were pooled and centrifuged at 12,800 g for 20 min. The resulting supernates were pooled and designated as cytoplasmic fraction. Human serum albumin (HSA) and phenylmethylsulfonylfluoride were added immediately to the cytoplasmic fraction at final concentrations of 1 mg/ml and 2 mM, respectively. This fraction was concentrated ~20 times by ultrafiltration over a UM2 membrane to a final volume of 5.0 ml and assayed.

The pellet containing crude membranous material was resuspended in 5.0 ml lysis medium and added to 15 ml of 60% sucrose in lysis medium in a 35-ml capacity Beckman ultracentrifuge tube. After vigorous mixing, 15 ml of 35% sucrose in lysis medium was carefully overlaid. This 35%–45% discontinuous sucrose gradient was centrifuged at 500,000 g for 30 min in a T160 fixed-angle rotor. The supernate was removed, dialyzed against PBS, and assayed. A 200-μl aliquot of each dialyzed sample was added to 10.0 ml of Bray's solution and counted for 10 min in a scintillation counter to measure the total counts per minute (cpm) incorporated into released proteins.

**Subcellular Localization and Characterization of CSA Activity**

Two milliliters of concentrated cytoplasmic fraction and 2.0 ml of stage III CM were separately chromatographed on a Sephacryl-200 column (1.5 × 50 cm) equilibrated in 0.03 M Tris-HCl, pH 7.4. Fractions were collected and assayed for CSA. The plasma membrane fraction was incubated for 4 hr at 4°C after vigorous vortexing with 5.0 ml 4 M guanidine-HCl in PBS. After incubation, the membranes were centrifuged for 1 hr at 45,000 g. The supernate was saved, and the pellet was resuspended by vortexing in 5.0 ml of 0.3 M lithium iodosalicylate in 0.05 M Tris-HCl, pH 7.8.\textsuperscript{40} The centrifugation was repeated and the pellet was extracted in an identical manner with 5.0 ml 0.5% Triton X-100 in PBS and finally with 0.5 ml 1% sodium dodecyl sulfate (SDS). Before assaying, these plasma membrane elutions were dialyzed against PBS at 4°C. Since Triton X-100 and SDS are not readily dialyzable, Triton X-100 was removed by adsorption to SM2 polystyrene Biobeads. The SDS was removed by the method of Weber and Kuter.\textsuperscript{41} Crude GCT CM treated with comparable concentrations of Triton X-100 and SDS served as controls for the possible toxic effects of residual amounts of these membrane-solubilizing agents on colony growth.

**RESULTS**

**Fractionation and Properties of GCT Cell CM**

A summary of the procedures utilized in the partial purification of CM is shown in Table 1. Ultrafiltration followed by calcium phosphate gel adsorption led to a tenfold purification of CSA. The results of various treatments on this stage III CSA are shown in Table 2. H-CSA showed greater resistance to inactivation by α-chymotrypsin than M-CSA, whereas M-CSA was more resistant to trypsin. Both were largely inactivated by pronase. M-CSA and H-CSA were resistant to DNAase, RNAase, neuraminidase, lipase, and phospholipase-C (data not shown).

M-CSA and H-CSA were both inactivated by periodate treatment at concentrations greater than 50 mM. At 0.5 mM, only M-CSA was partially inactivated. M-CSA and H-CSA were both unaffected after exposure of CM at 56°C for 2 hr. Treatment at 75°C resulted in a 75% reduction in M-CSA and only a 15% reduction of H-CSA. Although CM exposed to 90°C for 2 hr did not support human marrow colony growth, it did support the growth of clusters. Denaturation, reduction, and alkylation, by themselves, had no effect on either H-CSA or M-CSA. However, reduction with dithiothreitol (DTT) followed by alkylation with N-ethylmaleimide (NEM) completely neutralized both M-CSA and H-CSA in both native (untreated) and denatured stage III CM. Also, treatment with NEM alone resulted in a small but significant loss of both H-CSA (32%) and M-CSA (31%).

**Fractionation of Stage III CM**

Stage III CM was fractionated on a DEAE cellulose column. The results are shown in Fig. 1. The majority of both M-CSA and H-CSA eluted at the end of the NaCl gradient (apparent NaCl molarity of 0.2–0.25 M). This step resulted in a 40-fold increase in
Table 1. Summary of Fractionation of M-CSA and H-CSA From GCT CM

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Total Protein (mg)</th>
<th>Total Activity (10^4 x colonies)</th>
<th>Specific Activity (10^4 colonies/mg)</th>
<th>Purification (-fold)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Crude GCT CM</td>
<td>387.0</td>
<td></td>
<td></td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>Mouse</td>
<td>2.4</td>
<td>0.062</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>3.94</td>
<td>0.102</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>II Ultrafiltration and dialysis</td>
<td>155.0</td>
<td></td>
<td></td>
<td>3.0</td>
<td>120%</td>
</tr>
<tr>
<td>Mouse</td>
<td>2.88</td>
<td>0.186</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>4.56</td>
<td>0.30</td>
<td></td>
<td>2.94</td>
<td>116%</td>
</tr>
<tr>
<td>III Calcium phosphate gel</td>
<td>37.0</td>
<td></td>
<td></td>
<td>9.2</td>
<td>89%</td>
</tr>
<tr>
<td>Mouse</td>
<td>2.13</td>
<td>0.576</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>3.07</td>
<td>0.830</td>
<td></td>
<td>8.15</td>
<td>78%</td>
</tr>
<tr>
<td>IV DEAE-cellulose</td>
<td>4.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>1.20</td>
<td>2.78</td>
<td></td>
<td>45</td>
<td>50%</td>
</tr>
<tr>
<td>Human</td>
<td>1.82</td>
<td>4.22</td>
<td></td>
<td>41.5</td>
<td>46%</td>
</tr>
<tr>
<td>V Sephacryl S-200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMW mouse</td>
<td>0.41</td>
<td>0.48</td>
<td>11.6</td>
<td>187</td>
<td>20%</td>
</tr>
<tr>
<td>LMW human</td>
<td>0.41</td>
<td>1.26</td>
<td>30.6</td>
<td>300</td>
<td>32%</td>
</tr>
<tr>
<td>(A) Preparative isoelectric focusing LMW-CSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>0.03</td>
<td>0.100</td>
<td>30.6</td>
<td>490</td>
<td>4.2%</td>
</tr>
<tr>
<td>Man</td>
<td>0.03</td>
<td>0.236</td>
<td>76.5</td>
<td>750</td>
<td>6.0%</td>
</tr>
<tr>
<td>(B) Preparative polyacrylamide gel electrophoresis of LMW-CSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>0.011</td>
<td>0.063</td>
<td>55.1</td>
<td>880</td>
<td>2.6%</td>
</tr>
<tr>
<td>Man</td>
<td>0.0093</td>
<td>0.122</td>
<td>112.0</td>
<td>1,100</td>
<td>3.0%</td>
</tr>
</tbody>
</table>

specific activity. The active pool was applied to a Sephacryl S-200 column. M-CSA eluted heterogeneously from S-200 in two areas with apparent molecular weights of 145,000 daltons (HMW-M-CSA) and 40,000 daltons (LMW-M-CSA) (Fig. 2). The H-CSA cochromatographed with the LMW-M-CSA. At this point there was a 300-fold purification of H-CSA (Table 1).

When ^1^C-PEG-CM or ^3^H-PEG-CM were similarly purified, the major biosynthetic product of GCT cells appeared to be a fucose containing glycoprotein with apparent subunit molecular weight on SDS polyacrylamide gels of 220,000–230,000 daltons. Fibronectin, the major cell surface constituent of fibroblasts and certain other cultured cells, has a similar subunit molecular weight and biochemical properties.

![Fig. 1. Chromatography of concentrated stage III GCT CM on DEAE cellulose; details are given in text. One-tenth milliliter of each fraction (10 ml/fraction) was diluted 1:10 with 10% FCS, and 0.1 ml was assayed against 10⁶ nonadherent human bone marrow (HBM) cells and 7.5 x 10⁶ mouse bone marrow (MBM) cells.](image-url)
the elution profile of CSAs showed little change when the S-200 column was run in denaturing conditions. The molecular weights actually appeared to increase slightly, an artifact that has been previously described. Elution of separately pooled and concentrated CSAs produced similar results. Gel filtration of stage IV CM on an Ultrogel AcA54 column also demonstrated HMW-M-CSA and the coelution of LMW M-CSA and H-CSA. As shown in Fig. 3B, the apparent molecular weight of H-CSA was about 30,000 daltons by this method.

The structure of the stage V CSA was further examined by fractionating 2.0-ml aliquots of the HMW-M-CSA and LMW-CSA pools on Con A-Sepharose affinity columns. The results are shown in Fig. 4(A and B). All of the LMW-CSA was found in the nonbinding fraction, and no LMW M-CSA or H-CSA could be detected in the elution. Eluted samples did contain an activity that stimulated human marrow cluster formation. The HMW-M-CSA, on the other hand, partially bound to Con A-Sepharose and could be eluted with amm.

LMW stage V CSA was further fractionated to determine if M-CSA and H-CSA molecules found in this pool could be biochemically separated. Prepara-

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**Fig. 2.** Gel filtration of pooled stage IV GCT CM on Sephacryl S-200. Each fraction was diluted 1:10 with 10% FCS, and 0.1 ml was assayed against 7.5 x 10⁶ mouse and 10⁷ human bone marrow cells. The elutions of standard proteins are shown by the arrows. High molecular weight M-CSA (HMW-M-CSA) and low molecular weight (LMW) CSAs were separately pooled and concentrated as described in Materials and Methods.

Since glycoproteins and some CSAs can behave anomalously on molecular sieves, the molecular weights of both HMW-M-CSA and LMW-CSA were reexamined by chromatographing stage IV CM on an identical S-200 column equilibrated in 6 M urea and on an Ultrogel AcA54 column. As shown in Fig. 3A,

**Fig. 3.** Gel filtration of 2.0 ml stage IV GCT CM on (A) Sephacryl S-200 equilibrated in 6 M urea and (B) Ultrogel AcA54 equilibrated in 0.03 M Tris-HCL, pH 7.4. The eluted fractions were dialyzed against PBS, and 0.1 ml of each fraction was assayed against 7.5 x 10⁶ MBM cells and 10⁷ HBM cells. Elutions of molecular weight markers are shown.

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**Fig. 4.** Fractionation of stage V GCT LMW-CSA (A) and HMW-M-CSA (B) on concanavalin-A-Sepharose columns as stated in Materials and Methods. α-Methylmannoside (amm) elution was begun at tube 20 in each column. The number of HBM colonies and MBM colonies stimulated by 0.1 ml of each fraction and absorbance at 280 nm are shown.
tive isoelectric focusing of pooled stage V LMW-CSA showed that both M-CSA and H-CSA focused in a broad heterogeneous fashion with approximate isoelectric points of 4.3 and 5.0, respectively (Fig. 5). Although there was a minor amount of H-CSA, which cofocused with M-CSA, the bulk of the two LMW-CSAs were separated by isoelectric focusing. In addition, although the yield was low, this step resulted in H-CSA purified 750-fold with respect to the crude CM (Table 1). Likewise, LMW-M-CSA and H-CSA could be partially separated when the stage V LMW-CSA pool was electrophoresed on preparative non-SDS gradient polyacrylamide slab gels (Fig. 6). Although this step also resulted in a low yield, H-CSA was purified approximately 1100-fold relative to the crude CM (Table 1). Crude 14C-CM, initially mixed with cold CM, was copurified with the bulk of unlabeled CM. After purification, the active fractions were run on 12% SDS polyacrylamide gels, revealing multiple radiolabeled bands when radioautographs of these gels were developed for 8 wk. This fact, coupled with the presence of several faint unlabeled Coomassie brilliant blue staining bands in the final preparation, indicated the lack of homogeneity of the final semipurified samples of LMW M-CSA and H-CSA.

Inhibition of Protein Synthesis by Cycloheximide or Serum Deprivation

Since GCT cells continue to produce copious amounts of CSA for 3–4 wk after exchange into serum-free conditions,23 the effects of cycloheximide and serum deprivation on protein synthesis and CSA production were investigated. Either long-term serum deprivation or cycloheximide treatment for 4 days reduced the amount of nondialyzable, incorporated 14C-leucine detected in GCT CM, but did not reduce to the same degree the amount of M-CSA and H-CSA assayed in CM (Table 3). To determine if CSA released by serum-deprived or cycloheximide-treated cells was due to shedding of membrane-bound CSA or release of preformed intracytoplasmic CSA, GCT cells were fractionated into subcellular components that were assayed for CSA.

Subcellular Compartmentalization of GCT CSA

Neither PBS washes of whole GCT cells nor the nuclear-mitochondrial fraction contained detectable CSA, whereas the cytoplasmic fraction contained significant amounts of M-CSA and H-CSA (Table 4). Purified plasma membranes prepared on discontinuous...
Table 4. CSA Derived From Subcellular Fractions of GCT Cells

<table>
<thead>
<tr>
<th>Marrow Growth</th>
<th>No. Colonies/10^6 Human Cells</th>
<th>No. Colonies/7.5 x 10^6 Mouse Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCT cell wash</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nuclear-mitochondrial fraction</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytosol fraction</td>
<td>155</td>
<td>140</td>
</tr>
<tr>
<td>Plasma membrane fraction*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PBS wash of membranes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6M guanidine-HCl elution</td>
<td>34 Clusters</td>
<td>28 Clusters</td>
</tr>
<tr>
<td>0.03 M lithium iodosalicylate elution</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5% Triton X-100 elution†</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1% SDS elution†</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

All samples were adjusted to 5.0 ml by ultrafiltration with an Amicon PM 10 filter.

*Crude GCT CM and plasma membrane elutions were treated with the above agents and were removed by dialysis and by the method described in the text before assaying.

†The same concentration of Triton X-100 in GCT CM reduced the number of colonies stimulated by 10% CM to 60% of control.

‡The same concentration of SDS in GCT CM reduced the number of colonies stimulated by 10% CM to 22% of control. Treatment of GCT CM with PBS, lithium iodosalicylate, and guanidine-HCl resulted in 100% recovery of CSA.

Fractionation of Cytoplasmic GCT CSA

The cytoplasmic fraction (~100 ml) stabilized with HSA and phenylmethylsulfonyl fluoride (final concentrations: 1 mg/ml and 2 mM, respectively) was concentrated by ultrafiltration (PM 10) to 2.0 ml and applied to a Sephacryl S-200 column (1.5 x 50 cm). For comparison, 2.0 ml of stage III CM were separately applied, and the results are shown in Fig. 7. Cytoplasmic CSAs have similar apparent molecular weights on S-200 as their secreted counterparts.

Morphology of Stimulated Marrow Cells

Various concentrations of crude CM and stage V LMW-CSA and HMW-M-CSA were incubated with mouse and human marrow for 7 and 14 days, respectively. At least 30 colonies per plate were removed and stained. The results are shown in Table 6. Crude GCT CM stimulated primarily neutrophil or mixed neutrophil-macrophage marrow colonies (GM-CSA). The percentage of neutrophilic colonies increased with increasing concentrations of crude CM. Stage V LMW-CSA stimulated a similar distribution of human and mouse marrow colony types as crude CM, and the percentage of neutrophilic colonies was also dependent on the concentration of the LMW-CSA. HMW-M-CSA at all concentrations tested, up to 20-fold concentrated, stimulated nearly 100% macrophage colony growth in the mouse, yet failed to stimulate any colony growth in human marrow.

Human eosinophilic colonies had a distinctive macroscopic appearance consistent with that described by Nissen-Druey et al. and were not mixed with either monocytes or granulocytes. Their identity...
was confirmed by the easily visualized blue-green color in the cytoplasm of maturing eosinophils stained with Luxol fast blue. The percentage of eosinophilic colonies present when human marrow was stimulated with 0.1 ml of crude CM increased from <5% at day 7 to ~20% at day 14 to ~40−50% at day 18. GCT CM did not stimulate the formation of eosinophil colonies in the mouse. The percent of human eosinophil colonies present at day 14 (20%) appeared independent of the concentration or purification stage of GCT H-CSA.

Several additional observations were made. In agreement with other observers using different sources of H-CSA, the number of small day-7 human marrow colonies stimulated by GCT CM were approximately 1−3-fold greater than the larger day-14 human colonies. In spite of this difference, the percentages of granulocytic, mixed, and macrophagic colonies were similar to that of day-14 colonies except that no eosinophilic colonies could be detected at 7 days (data not shown). Also, it should be noted that there were no obvious differences in the types of human marrow colonies stimulated by GCT CM derived from various stages of purification, except that the size of colonies decreased with increasing purification. There was also no evidence that GCT eosinophilic CSA (EO-CSA) could be separated from the bulk of GCT GM-CSA.

**Table 6. Morphology of Human and Mouse Marrow Colonies Stimulated by Crude GCT CM and by Semipurified GCT CSAs**

<table>
<thead>
<tr>
<th>Amount CSA</th>
<th>Source of CSA</th>
<th>Marrow Source</th>
<th>Eosinophilic</th>
<th>Neutrophilic</th>
<th>Macrophagic</th>
<th>Macrophagic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ml</td>
<td>Concentrated stage I GCT CM</td>
<td>Mouse</td>
<td>0</td>
<td>30</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td></td>
<td>16</td>
<td>38</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>0.01 ml</td>
<td>Concentrated stage I GCT CM</td>
<td>Mouse</td>
<td>0</td>
<td>16</td>
<td>54</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td></td>
<td>22</td>
<td>14</td>
<td>16</td>
<td>48</td>
</tr>
<tr>
<td>0.1 ml</td>
<td>LMW stage V GCT CM</td>
<td>Mouse</td>
<td>0</td>
<td>40</td>
<td>42</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td></td>
<td>20</td>
<td>42</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>0.01 ml</td>
<td>LMW stage V GCT CSA</td>
<td>Mouse</td>
<td>0</td>
<td>22</td>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td></td>
<td>18</td>
<td>20</td>
<td>26</td>
<td>36</td>
</tr>
<tr>
<td>0.1 ml</td>
<td>HMW stage V GCT M-CSA</td>
<td>Mouse</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>0.01 ml</td>
<td>HMW stage V GCT M-CSA</td>
<td>Mouse</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>94</td>
</tr>
</tbody>
</table>

Colonies were removed and stained after 7 days (mouse) or 14 days (human) culture. At least 30 colonies were analyzed from each plate examined. 7.5 x 10⁴ mouse marrow cells or 1 x 10⁶ nonadherent human marrow cells were cultured per plate. Colony number/sample ranged from 33 to 82 for mouse marrow and from 37 to 104 for human marrow.
macrophagic colony growth, and a 30,000-dalton activity, which stimulated primarily neutrophil and mixed neutrophil-macrophage colony growth. Their size and effects are similar to mouse CSAs released by human and mouse macrophages.22,25 At the concentrations used in these studies, no H-CSA could be detected in the HMW-M-CSA fraction. Other workers have shown that a high molecular weight component in medium conditioned by H-CSA-producing cells can stimulate human macrophagic cluster formation in vitro.24

The molecular weight of GCT H-CSA is similar to other H-CSAs purified from human lung,24 placenta,11 and leukocytes.19 With the exception of the ~100,000-dalton H-CSA released from a T-lymphoblastoid cell line,24 all H-CSAs thus far, including GCT H-CSA, have failed to bind to Con A-Sepharose. With the exceptions of human placenta and a T-lymphoblastoid cell line, all human sources of CSA also support mouse marrow growth. GCT HMW and LMW M-CSAs are antigenically related, since antihuman urinary CSF is able to neutralize 100% of the M-CSA in stage III GCT CM, which contains both species of GCT M-CSA.23 Antigenic differences between LMW-M-CSA and H-CSA are likely since the latter is not neutralized at any antisera dilution25. Similar antigenic differences have been demonstrated between M-CSA and H-CSA molecules released by human monocytes.53 The difference in the apparent molecular weights of HMW-M-CSA and LMW-M-CSA could also be due to carbohydrate differences. Other CSA glycoproteins have been shown to behave anomalously on molecular sieves.18 The relative elution of HMW M-CSA did not change when the S-200 column was equilibrated with a denaturing agent, suggesting that HMW-M-CSA was not an aggregate of LMW-M-CSA molecules.

LMW-M-CSA and H-CSA cochromatographed on Sephacryl S-200 but could be partially separated by preparative isoelectric focusing and preparative gradient polyacrylamide gel electrophoresis. This is consistent with the antisera neutralization data, suggesting antigenic difference between GCT M-CSA and H-CSA.23 Stanley34 has recently examined several murine and human CSAs, including those in a sample of our GCT CM, by means of a radioimmunoassay (RIA) that detects a specific subclass of CSA molecules that stimulate macrophagic colony growth in the mouse. None of the CSAs from human sources, except human urinary CSF, were detected in this RIA. It is of interest that HMW-M-CSA contained in crude GCT CM was not detected in this RIA, since it is a potent stimulator of macrophagic colony growth in the mouse. The antisera used in this RIA may thus be largely recognizing determinants that are specific for species rather than function (macrophage stimulator) determinants.

The purification of GCT LMW-M-CSA and H-CSA molecules in this study was 890- and 1100-fold, respectively, yielding specific activities of $5 \times 10^4$ colonies/mg for M-CSA and $1.0 \times 10^6$ colonies/mg for H-CSA. In spite of this high specific activity, the final preparation was found to be heterogeneous.

The presence of preformed CSA in cells has been controversial.55-57 The subcellular localization of GCT CSA indicates that GCT cells do contain a small pool of preformed CSA and that the majority is associated with the cytosol. The small amount of CSA found loosely associated with GCT plasma membranes may be due to CSA that is in the process of being exported outside of these cells. These results are consistent with the model of synthesis and export of glycoproteins proposed by Schachter and Rodin.58 Macrophage growth factor (L-cell CSA) has also been shown to be transiently associated with L-cell membranes during transport to the extracellular medium.59 On the other hand, Price, Till, and coworkers have reported that H-CSAs derived from human peripheral blood cells are associated with the plasma membranes of these cells and can be partially neutralized by antisera directed against other cell membrane components, namely, $\beta_2$-microglobulin and the cell line histocompatibility heavy chain antigen.60,61 The cytosol localization of GCT CSAs could be a peculiarity of this malignant cell line. Alternatively, the apparent membrane association of CSAs in mixed leukocyte homogenates could reflect binding of the molecules to nonproducing cells. Methodological differences might also account for the conflicting results.

Several glycoproteins as well as peptide hormones undergo post-translational modification before extracellular transport occurs, usually by enzymatic cleavage of N-terminal segments from precursor or prohormone molecules. GCT intracellular CSAs had apparent molecular weights similar to those of their secreted counterparts, suggesting that if modification of a pro-CSA molecule occurs, only a few amino acids are removed. Molecular sieves may not detect subtle differences in molecular weights between cytosol and secreted CSAs. We cannot exclude the possibility that a hypothetical precursor was specifically modified during the isolation procedure.

GCT CM did not stimulate eosinophilic colonies in the mouse, but this is not surprising since mouse EO-CSA has thus far been derived only from lymphocytes or activated lymphocytes.62-64 GCT cells did release an ~30,000-dalton human EO-CSA that copurified with the human GM-CSA, suggesting the possi-
bility that these two CSAs might be the same molecule and identical or similar to a 30,000-dalton GCT product that induced the proliferation and differentiation of a population of acute myelogenous leukemia (AML) cells grown in vitro. However, human placenta CM contains similar 30,000-dalton GM and EO-CSAs that can be at least partially separated from each other by using hydrophobic chromatography. GM-CSA and EO-CSA derived from mouse lymphocyte CM could be separated by starch gel electrophoresis. These activities possessed antigenic differences, since antiserum to mouse lung GM neutralized mouse GM CSA but not EO-CSA. Thus, although we were not able to separate these molecules, other studies suggest that EO-CSA and GM-CSA are distinct molecules.

GCT CM stimulated two populations of human CFU-C, one giving rise to day-7 GM colonies and the other giving rise to day-14 GM and eosinophil colonies. The day-14 GM colonies were much larger and contained similar percentages of mature neutrophils as day-7 colonies, suggesting that the day-14 colonies were derived from a more immature CFU-C. Human lung has also been found to release two electrophoretically distinct human GM CSAs, one stimulating day-7 colonies and the other day-14 colonies. The CFU-C target cells for these factors were partially separated by velocity sedimentation. At present, the GCT H-CSAs stimulating day-7 and day-14 colonies have not been separated.

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REFERENCES

28. Nicola NA, Metcalf D, Johnson GR, Burgess AW: Prepara-
34. Davis BJ: Disc electrophoresis. III. Methods and application to human serum proteins. Ann NY Acad Sci 121:404, 1964
55. Russett FW, Chervenick PA: Regulation of the release of colony stimulating factor from monocytes by endotoxin and polyinosinic polycytidylic acid. J Lab Clin Med 83:64, 1974
The fractionation, characterization, and subcellular localization of colony-stimulating activities released by the human monocyte-like cell line, GCT

JF DiPersio, JK Brennan, MA Lichtman, CN Abboud and FH Kirkpatrick