Purification of Two Forms of Colony-stimulating Factor from Mouse L-cell-conditioned Medium*

A modified procedure for the purification of the colony-stimulating factors (CSFs) in mouse L-cell-conditioned medium is used to isolate two forms of CSF, which are separable by reversed-phase high performance liquid chromatography with 300-A pore size supports. The specific biological activity of these CSFs (2 x 10⁹ colonies/mg) was considerably higher than has been achieved by other methods. Even at high concentration (200 pm) both molecules stimulated predominantly more macrophage than granulocyte colonies; however, the less hydrophobic form appeared to stimulate the formation of more pure granulocytic colonies. Almost twice as much of the less hydrophobic CSF was recovered from L-cell-conditioned medium. Analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that both forms of L-cell CSF had apparent molecular masses of approximately 70,000 daltons. However, on reduction with 2-mercaptoethanol, while both forms generated a 39,000-dalton subunit, the less hydrophobic form also yielded a 32,000-dalton subunit. Storage of either form of L-cell CSF at pH 2.1, in the presence of acetonitrile or isopropanol, destroyed the biological activity. Electrophoretic analysis of the L-cell CSFs stored under these conditions indicated that this was associated with a spontaneous dissociation of the CSF dimer into the inactive subunits. There was some charge heterogeneity (pl 3.5-4.7) indicating different degrees of glycosylation. The unique N-terminal amino acid sequences of both forms of CSF were the same: (Lys-Glu-Val-Ser-Glu-His-X-Ser-His-Met-Ile-Gly-Asn). Thus, the polypeptide chains appear to be identical for the subunits of both forms of L-cell CSF.

Granulocyte and macrophage production is known to be regulated by a set of glycoproteins, the colony-stimulating factors (CSFs) (1-4). At least four different CSFs, active in stimulating granulocyte and macrophage proliferation, have been purified to apparent homogeneity: granulocyte-macrophage (GM)-CSF from mouse lung-conditioned medium (CM) (1), macrophage (M)-CSF (also called CSF-1, Ref. 2) from mouse L-cell CM (2, 5), G-CSF from mouse lung CM (3) and multi-CSF (also called IL-3, Ref. 6) from WEHI-3B(D') CM (4, 6), or pokeweed mitogen-stimulated spleen cell CM (7). The last of these molecules (multi-CSF) is known to stimulate the production of many different blood cells in addition to granulocytes and macrophages (8).

The molecular and antigenic properties of GM-CSF from mouse lung CM (1, 9, 10) and G-CSF (11, 12) from endotoxin serum (11) are distinct from M-CSF (12) purified from mouse L-cell CM (2, 5). Whereas GM-CSF and G-CSF appear to be single chain glycoproteins with apparent molecular weights of 20-25,000 (1, 3), the M-CSF purified from L-cell CM is a dimer (molecular weight 70,000) which is only active in the dimeric form (2). Interestingly, the antibodies against M-CSF purified from L-cell CM (apparent molecular weight 70,000) neutralize the GM-CSF in endotoxin serum (a 23,000 molecular weight glycoprotein) (11, 12) but not G-CSF (11, 12) or GM-CSF from mouse lung CM (9). M-CSF has been purified from L-cell CM by at least two methods (2, 5) and while there is general agreement about the molecular properties of M-CSF, the cellular binding specificities of the different preparations appear to be different (13, 14). 125I-labeled M-CSF, affinity purified from mouse L-cell CM (5) appears to bind to both macrophages and granulocytes (14), whereas the M-CSF prepared by gel filtration, ion-exchange, and lectin chromatography (2) appears to bind only to macrophages or their precursors (15).

In this study we have developed a high resolution purification procedure for obtaining two forms of CSF from mouse L-cell CM (2, 15). It has been reported earlier that unfractionated L-cell CM was able to stimulate both macrophage and mixed granulocyte macrophage bone marrow colonies (17). Our semi-solid bone marrow bioassays (18) indicated that unfractionated mouse L-cell CM was able to stimulate the production of granulocyte, macrophage, and mixed GM colonies. Although several molecular forms of CSF have been observed in L-cell CM (19) the biological specificity and molecular properties have only been examined in detail for one form CSF-1 (2). Those studies reported that CSF-1 only stimulated the formation of macrophage colonies. There is at

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§ To whom correspondence should be sent.

The abbreviations used are: CSF, colony-stimulating factor; CM, conditioned medium; GM, granulocyte-macrophage; G, granulocyte; M, macrophage; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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least one report of the partial purification of a high molecular weight CSF from a cloned cell line which had an apparent molecular weight of 68,000 but which was still able to stimulate both granulocyte and macrophage colonies (20). Provisions to the purification method were aimed mainly at improving the recovery and speed of the preparation of L-cell M-CSF, although the inclusion of reversed-phase high performance liquid chromatography (RP-HPLC) was also designed to increase the chromatographic resolution of the final step. This procedure has resulted in the characterization and purification of two forms of CSF from mouse L-cell CM.

**MATERIALS AND METHODS**

The protein content of the conditioned medium and CSF pools from Stages 1-4 of the purification was measured by the Lowry procedure (21).

**Production of Serum-free L-cell CM (Stage 1)—** Serum-free L-cell CM was prepared from mouse L-cells according to the procedure published previously (16), excepting that the cells were grown in large flat-bottomed glass flasks (surface area approximately 600 cm²) instead of roller culture bottles. This method of producing the L-cell CM was novel and efficient and cost effective than the roller cultures. The cells remained attached for the duration of the serum-free cultures and could be used to prepare several batches of conditioned medium.

**Calcium Phosphate Gel Adsorption (Stage 2)—** As described previously (15), calcium phosphate gel (22) was stirred into 20 liters of the serum-free L-cell CM (1 ml of gel/4 mg of total protein) and allowed to settle. After 1 h, the gel was resuspended by stirring with a glass rod and allowed to settle for at least 4 h. Part of the supernatant fluid was decanted, and the calcium phosphate gel was collected by centrifugation (10,000 × g for 15 min). The gel pellet was washed twice with an equal volume of 0.03 M sodium phosphate buffer (pH 6.5), followed by three washes with an equal volume of 0.1 M sodium phosphate buffer (pH 6.5, containing sodium azide 0.02%, w/v, and polyethylene glycol 6000, 0.005%, w/v). The subsequent purification steps (excepting the RP-HPLC) were performed in a cold cabinet below 8°C. The buffers for the conventional columns contained sodium azide (0.02%, w/v) and polyethylene glycol 6000 (0.005%, w/v).

**DEAE-Sepharose CL-6B Chromatography (Stage 3)—** The active fractions eluted from calcium phosphate gel with sodium phosphate buffer (pH 6.5, 0.1 M) were pooled and exchanged into Tris-HCl buffer (pH 7.4, 0.1 M) by dialysis. The dialyzed sample was applied to a column (1.5 × 26 cm) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with the same buffer. After washing the sample through with 250 ml of this buffer, the CSF was eluted by adding NaCl (0.5 M) to the buffer.

**Ultralow AcA44 Chromatography (Stage 4)—** The active fractions from the DEAE-Sepharose CL-6B column were pooled, concentrated to 10 ml over a YM-10 membrane (Amicon) using an Amicon TCF-10 and an Amicon microconcentrator and chromatographed on an Ultralow AcA44 (LKB) column (2.6 × 92 cm) equilibrated with Tris-HCl (0.03 M, pH 7.4) at 13 ml/h.

**Concanaval A Sepharose Chromatography—** For some analytical experiments, aliquots of the active fractions from the Ultralow AcA44 column were concentrated, dialyzed against sodium acetate buffer (0.1 M, pH 6.0 containing NaCl (0. M), divalent metal ions MgCl₂, CaCl₂, and MnCl₂ (1 mM)), and chromatographed on a concanaval A Sepharose 4B (Pharmacia) column (1.5 × 25 cm) as described previously (2, 15). The glycoproteins were eluted at 6 ml/h using a mixture of α-methyl glucopyranoside (0.05 M) and α-methyl mannoside (0.05 M) in the starting buffer.

**High Performance Liquid Chromatography (HPLC)—** HPLC on conventional columns was performed at room temperature using a Beckman model 324-40 liquid chromatograph fitted with a 2-ml injection loop. Samples were usually loaded by pumping directly through the primary solvent pump. Proteins were monitored using a LDC spectromonitor III or a Beckman 164 detector and a Kratos FS950 fluorimeter (fitted with 214 nm excitation, 340 nm emission filters). The amount of protein was estimated by comparison with the absorption of a known amount of bovine serum albumin at the same wavelength.

**Size Exclusion on TSK3000 SW (Stage 5)—** Fractions from the AcA44 column containing biological activity were pooled (200 ml) and concentrated over a YM-10 membrane to a final volume of 3 ml. The concentrate was then chromatographed, in 500-μl aliquots, on a TSK3000 SW size exclusion column (30 cm × 7.5 mm, internal diameter, Tosoh Soda Manufacturing Co., Japan) using a mobile phase of 0.05 M sodium chloride, 0.09% (v/v) SDS, 0.1% (v/v) phenol, pH 7.4, at a flow rate of 1 ml/min at ambient temperature. Proteins eluting from the column were detected by ultraviolet absorption at 215 nm (Beckman model 164 detector). Fractions (1 ml) were collected automatically (Pharmacia FRAC100 fraction collector) into polypropylene tubes containing 10 ml of a 2% (v/v) Tween 20 solution (Sigma). Aliquots (1 μl) were taken for biological assay to locate the CSF for further purification on RP-HPLC.

**Reversed-phase HPLC on Ultrapure RPSC (Stage 6)—** CSF-containing fractions from the TSK3000 SW column were pooled (6 ml) for further purification on an Ultrapure RPSC column (7.5 × 7.6 mm, internal diameter, 300-Å pore size, C8 bonded phase, Beckman Instrument Co.) (34). An aliquot (2 ml) of the pool was loaded directly onto the column using an Altex model 210 rotary injection valve fitted with a 2-ml sample loop. Proteins were eluted, in order of increasing relative hydrophobicity, at ambient temperature, and a flow rate of 1 ml/min with a linearly increasing gradient (1% min) between the primary solvent (aqueous sodium chloride 0.9%, w/v, adjusted to pH 2.1 with HCl) and the secondary organic modifier, acetonitrile. Proteins eluting from the column were detected by sequential ultraviolet absorption (215 nm) and endogenous tryptophan fluorescence (214-nm excitation, 340-nm emission) (21). Fractions (1 ml) were collected directly into tubes containing 100 μl of sodium phosphate (0.1 M, pH 7.8) and Tween 20 (0.2%, w/v), yielding a final sample pH of approximately 6.5. Aliquots (1 μl) of these fractions were taken for assay of CSF.

**Rechromatography on Ultrapure RPSC (Stage 7)—** After dilution with the primary solvent (1:1) the fractions containing CSF (48-59 min) were rechromatographed. The much reduced amount of protein allowed the complete separation of the CSFs from the contaminating proteins.

**Microce RP-HPLC on Brownlee Aquapore RP-300 Column (Stage 8)—** The final purification of the L-cell CSF was accomplished by microscale RP-HPLC (36). This was performed on a short Brownlee Aquapore RP-300 microbore "guard column" (3 cm × 2.1 mm, internal diameter, 300-Å pore size C8 bonded phase, Brownlee, CA) using a Hewlett-Packard model 1090 liquid chromatograph. A Rheodyne model 7125 injection valve fitted with a 2-ml injection loop inserted into the column oven compartment immediately prior to the column was used to load aliquots of the diluted pool (1:1 with primary buffer containing 0.02% Tween 20) from Stage 7. Proteins were eluted from the column using a 60-min linear gradient from aqueous trifluoroacetic acid (0.15%, v/v) to acetonitrile (60%, v/v)/aqueous trifluoroacetic acid (0.15%, v/v) at 1 ml/min. The initial fraction was 100 μl/min and the column temperature 45°C. Elution profiles were monitored simultaneously at 210, 254, and 280 nm using a Hewlett-Packard model 1040 diode array detector and the spectral data (200-320 nm in 2-nm increments) stored on disc for subsequent analysis. The column eluate was collected manually (and an allowance was made for the dead volume between the detector and the collection tube). The CSF was recovered in 60-100 μl of buffer and applied directly to a glass disc for sequence analysis, or the fraction was neutralized to pH 6.5 with 10 μl of sodium phosphate buffer (0.1 M, pH 7.8, containing Tween 20, 0.2%, v/v) and stored at −20°C.

**Internal Amino Acid Sequence Determination—** Automated amino acid sequence analysis (23) of the two forms of purified L-cell CSF was performed on an Applied Biosystems Sequencer (model 470A) fitted with a modified microconverter flask. Polybrene was used as a carrier (24). The phenylthiohydantoin amino acids were identified and quantitated by RP-HPLC (25).

**Isoelectric Focusing and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—** Polyacrylamide gel electrophoresis (10% acrylamide, w/v) in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described previously (1, 25) in the presence and absence of the reducing agent dithiothreitol (10 mM). Apparent molecular weights were estimated by comparison with the migration of two standard sets of proteins (Pharmacia, Sweden). Apparent molecular weights were estimated by extrapolation from the center of the stained protein or autoradiographed bands. Two-dimensional electophoretic analysis was performed using isoelectric focusing and SDS-PAGE as described previously (26). The gels were stained either with Coomassie Blue R250 (27) or silver (28, 29). The 125I-labeled proteins were detected by ultraviolet absorption at 215 nm (Beckman model 164 detector). Fractions (1 ml) were collected automatically (Pharmacia FRAC100 fraction collector) into polypropylene tubes containing 10 ml of a 2% (v/v) Tween 20 solution (Sigma). Aliquots (1 μl) were taken for biological assay to locate the CSF for further purification on RP-HPLC.
CSFs were analyzed by isoelectric focusing on agarose gels as described previously (30).

Bioassy of the CSFs—Assays for CSF were performed using 75,000 C57Bl bone marrow cells in semisolid agar medium (18) and colony morphology determined either by using aceto-orcein (31) or by staining of the whole agar cultures with Luxol Fast Blue and hematoxylin (32). Samples were sterilized by filtration (0.45 μ) before assay, and if the sample contained either isopropanol or acetonitrile the organic solvent was removed by gel filtration using a Sephadex G-25 column (Pharmacia, PD-10) equilibrated with normal saline. (The gel filtration step could be avoided provided that the final concentration of the organic solvent in the semisolid bone marrow cultures was no more than 0.05%, v/v.) Colonies resulting from CSF stimulation were counted after 7 days of incubation using a dissection microscope.

Radioiodination of Proteins—Aliquots of column RP-HPLC fractions (containing approximately 100 ng of protein) were reduced to 60 μl using a Speed-Vac rotary evaporator. This solution was made up to 0.125 mM KI, buffered using 40 μl of sodium borate (0.2 M, pH 8.4), and 1 μCi of 125I (Amersham, Bucks, United Kingdom) added. Radioiodination was effected by the method of Tejedor and Ballesta (33) for 30 min. The filter pad containing the NaCl and chloramintin T was replaced at 10 and 20 min. (It should be noted that for reproducible results it is important to dissolve the chloramintin-T in sodium borate buffer (0.05 M, pH 8.4)). The remaining free iodide was removed by exchanging the 125I-labeled protein into phosphate-buffered saline using a Sephadex G-25 column (PD-10, Pharmacia).

RESULTS AND DISCUSSION

The production of CSF by mouse L-cells and the initial calcium phosphate gel extraction yielded results similar to those published previously (2, 15). However, in four separate purifications we have observed an apparent increase in the total colony stimulating activity after absorption and elution of the activity from the calcium phosphate gel (Table I). This suggests that unfraccionated L-cell conditioned medium contains significant amounts of substances inhibitory for colony growth. In spite of the use of a smaller DEAE-Sepharose column and the batch elution with 0.5 M NaCl, the total degree of purification for the CSF (126-fold) (Table I) was similar to that obtained in earlier reports (2, 15). The DEAE-Sepharose step did not lead to a significant improvement in the specific activity of the CSF; however, the volume of the CSF was considerably reduced. Elution of the proteins using a salt gradient achieves further purification, but the CSF elutes as several broad peaks. We were aiming to recover all forms of CSF in L-cell CM; thus we used batch elution at this stage of the purification. Gel filtration on Ultrogel AcA44 (Fig. 1) yielded a further 4-fold purification (Table I). The L-cell CSFs eluted between 1450 and 1750 ml from this column. The center of the CSF peak could be pooled and a significantly improved purification obtained; however, we again decided to pool all of the biologically active fractions and effect the separation of different forms later in the purification procedure. These procedures reduced the protein content of a 20-liter batch of serum-free L-cell CM to 4.5 mg.

Concanavalin A Sepharose Chromatography—Initially, the next step of our purification procedure for L-cell CM CSF followed the published procedures (2, 15) by using concanavalin A Sepharose. Although glycoproteins (including some of the CSF) bind to concanavalin A Sepharose and can be eluted using α-methylglucoside, a considerable proportion of the CSF does not bind to the lectin column (19). The concanavalin A Sepharose procedure was difficult to control because the proteins eluted from the column with the sugars invariably contain significant amounts (1-10 μg/ml) of concanavalin A. Attempts to decrease the amount of contaminating concanavalin A by reducing the pH and using an acetate buffer system were not successful. The high salt pH 6 conditions reported earlier (2) appeared to be the best conditions for preparing L-cell CSF with low levels of contaminating concanavalin A. It is interesting to note that it has been reported that the L-cell CSF which failed to bind to concanavalin A appeared to be antigenically different (19). Only 40% of this CSF could be neutralized by an antiserum raised against the CSF eluted from concanavalin A Sepharose (19). Thus, this form of L-cell CM CSF appeared to be significantly different from the form bound to concanavalin A Sepharose. We considered that it was undesirable to discard up to 50% of the L-cell CSF; however, the concanavalin A binding CSF was significantly enriched, so we decided to use this material for our initial study of the properties of L-cell CSF on RP-HPLC.

![FIG. 1. Ultrogel AcA44 gel filtration of the CSF (B) concentrate from the DEAE-Sepharose CL6B column. The sample was mixed with blue dextran before chromatography. Much of the contaminating protein (e.g. albumin) bound to the blue dextran, causing these proteins to elute earlier. The elution position of the CSF (V/Vo = 2.2) was not effected by the blue dextran. Although a small amount of colony-stimulating activity was observed at apparently high molecular weight (V/Vo = 1.7) only the fractions associated with the major peak of biological activity (from 1450-1750 ml) were pooled for further purification.](https://example.com/fig1.png)

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Purification of CSFs from mouse L-cell CM</th>
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<tr>
<td>Stage</td>
<td>Total protein</td>
</tr>
<tr>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>1. L-cell-conditioned medium</td>
<td>396</td>
</tr>
<tr>
<td>2. Calcium phosphate gel</td>
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<tr>
<td>3. DEAE-Sepharose CL6B</td>
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<tr>
<td>4. Ultrogel AcA44 HPLC steps</td>
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<tr>
<td>5. TSK3000</td>
<td>4.0</td>
</tr>
<tr>
<td>6. RP-/C3/NaCl</td>
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</tr>
<tr>
<td>7. RP-/C3/NaCl</td>
<td>0.045</td>
</tr>
<tr>
<td>8. Microcore RP-HPLC*</td>
<td>Peak A</td>
</tr>
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<td></td>
<td>Peak B</td>
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</tbody>
</table>

* The total protein associated with the CSF at Stages 1-4 was calculated using the Lowry method. For the CSF from the HPLC procedures the total protein was calculated from the absorbance (at 215 nm) relative to bovine serum albumin.

*1 L-cell CM appears to contain inhibitor(s) of bone marrow colony formation which are removed by the calcium phosphate gel extraction. The yield of CSF at each stage was calculated from the titration of the unfraccionated L-cell CM.

*2 The microcore HPLC was performed on aliquots (100 μl) of the active fractions from Stage 7, but the data presented have been calculated by assuming that all of the CSF in Stage 7 is processed.

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RP-HPLC Purification and Stability of the CSF Which Binds to Concanavalin A Sepharose—The conditions for RP-
HPLC of the L-cell CM proteins, which had been eluted from concanavalin A Sepharose, were studied initially using a C3 Ultrapore RPSC column. The CSF was eluted using a gradient of isopropanol (1%, v/v, per min) in aqueous trifluoroacetic acid (0.2%, v/v) (Fig. 2). Several proteins separated from the region of biological activity (35-42 min), indicating that the semipurified L-cell CSF which we had eluted from concanavalin A Sepharose did not appear to be as pure as has been achieved by others (2, 15). Despite this, a major peak of colony-stimulating activity was obtained. More than 90% of the colonies stimulated by this CSF were composed solely of macrophages. At the high protein loadings used in this experiment (Fig. 2) the resolution of separate protein peaks was not complete; however, the specific activity of the CSF across the protein peak (Fig. 2) was not constant. Most of the biological activity was associated with the trailing edge of the protein peak, and the protein associated with the leading edge of this peak did not stimulate the formation of bone marrow colonies.

Analysis of the fractions associated with the major protein peak (Fig. 2) was performed using SDS-PAGE and isoelectric focusing/SDS-PAGE. However, attempts to detect 100-200 ng of protein in these fractions by Coomassie Blue staining (27) or by either of two different silver stain procedures (28, 29) were unsuccessful. In contrast, the set of standard proteins (Pharmacia, Sweden) and the different forms of GM-CSF purified from mouse lung-conditioned medium were detectable with the silver stain even at 10 ng. The low sensitivity of the silver-staining procedure for these L-cell CM glycoproteins which include CSF was presumably related to the amount of carbohydrate associated with these molecules (38, 39). Aliquots (100 µl) of each fraction were radiolabeled (33) before electrophoresis and the iodinated proteins detected using autoradiography.

The CSF protein migrates as a broad band even after removal of some of the carbohydrate (39). The molecular weights assigned to the dimer and monomers (see below) are measured from the center of the broad bands (Fig. 3A). It is still not clear why the CSF protein migrates as such a diffuse band; however, the charge heterogeneity (Fig. 3B) is associated with carbohydrate differences (39). The broadness of these bands may reflect the carbohydrate additions which cause different amounts of sodium dodecyl sulfate binding. Thus, the apparent molecular weights assigned to the dimer and monomers only approximately reflect the size of the subunits. The results indicated that the fractions from the leading edge of the major protein peak (fraction 37, Fig. 2) contained two proteins (apparent molecular weights 55,000 and 37,000, Fig. 3A, lane a) which in the presence of dithiothreitol yielded two proteins of apparent molecular weights 65,000 and 39,000 (Fig. 3A, lane b). The protein associated with the trailing edge of the major peak (fraction 49, Fig. 2) contained a major radiiodinated protein with an apparent molecular weight of 70,000 (Fig. 3A, lane c). On reduction this radiolabeled protein yielded a single polypeptide (apparent molecular weight 39,000, Fig. 3A, lane d). The two proteins on the leading edge of the major RP-HPLC peak (Fig. 2) were not associated with the CSF biological activity nor were their molecular properties consistent with the properties of biologically active CSF from L-cell CM. However, the protein on the trailing edge appeared to be the disulfide-linked dimer corresponding to L-cell CM CSF. (It is likely that the low molecular weight protein on the leading edge of the RP-HPLC peak was one of the subunits of the CSF dimer (see below).)

Neither the apparent molecular weight nor the biological activity of the unreduced purified L-cell CSF eluted from the reverse phase column with the isopropanol gradient were stable under our initial storage conditions. After 2 weeks of storage all of the biological activity of the pure CSF (see Fig. 3A, lane c) had disappeared completely. Two-dimensional electrophoretic analysis (isoelectric focusing/SDS-PAGE) of the CSF in fraction 39 (Fig. 2), in the absence of reducing agent, revealed that storage of the fraction for 1 week (i.e. at pH 2.1, in the presence of isopropanol) partially dissociated the CSF to its 39,000-dalton subunits (Fig. 3B). In the presence of denaturing agents (e.g. 40%, v/v, isopropanol) the individual subunits appear to unfold leading to a disruption of the dimer. Even under mild conditions native CSF can be reduced by mercaptoethanol; when the intrachain disulfides are reduced the dimer must become unstable. Presumably native CSF contains two polypeptide chains which form intrachain disulfide bonds, but not interchain disulfide bonds. The solvent denaturation was prevented by immediately adjusting the pH of the RP-HPLC fractions to pH 6.5 with 100 µl of phosphate buffer (0.1 M, pH 7.8, containing Tween 20, 0.2%, v/v).

Thus, it seems that the broad peak of protein associated with the CSF biological activity recovered from the reversed phase column (Fig. 2) was mainly a mixture of the CSF monomer and dimer. On dissociation to the monomer the CSF is inactivated and also eluted earlier from the reversed phase column. Even though the CSF protein is substantially purified by this procedure the extent of denaturation by the acidic isopropanol precludes the use of these conditions for the purification of biologically active CSF from L-cell CM.

It appears that subunit separation (and thus loss of biological activity) can occur by two distinct pathways. In the presence of isopropanol (or acetonitrile) at pH 2.1 the subunit interactions are reduced sufficiently to allow dissociation. Since disulfide bonds are stable under these conditions this shows that the subunits are not covalently linked. Dissociation in the presence of mercaptoethanol presumably occurs because the reduction of the disulfide bonds destabilizes the subunit conformation sufficiently to disrupt the interactions between subunits. At present the subunit interactions have only been analyzed using sodium dodecyl gel electrophoresis.

HPLC Purification of CSFs from L-cell CM—The loss of CSF and the concanavalin A contamination led us to replace the concanavalin A Sepharose chromatography by a sequence of size exclusion HPLC and RP-HPLC procedures. Thus, this HPLC protocol allowed the purification of all of the CSF species present in L-cell CM. The fractions from the Ultrogel AcA44 column which contained CSF were concentrated and

Fig. 2. Preparative RP-HPLC of L-cell CM CSF previously eluted from concanavalin A Sepharose. The isopropanol gradient (0-80% at 1 ml/min) yielded a single major protein (——) peak which was broad but not coincident with the CSF activity (■).

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Purification of Two Forms of M-CSF from L-cell CM

The two proteins associated with the CSF activity eluted from the RPSC column contained minor amounts of contaminating proteins; however, rechromatography under identical conditions (Fig. 6) separated these from the L-cell CM CSFs. Again two forms of L-cell CM CSF were partially separated on the Ultrapore RPSC column (eluting at 51 and 53 min, respectively (Fig. 6)). These could be completely separated by chromatographing 100-μl aliquots of the active peaks (Fig. 6) on a microbore HPLC system fitted with a C8, 300-Å column (Fig. 7). In the presence of trifluoroacetic acid the two forms of CSF eluted from this column at 52 and 54 min, respectively. There appeared to be approximately twice as much of the earlier eluting form of CSF (Fig. 7). For sequence analysis, the microbore fractions containing the CSF were immediately applied to the treated glass disk of the gas phase Sequencer. Again, when the CSF was to be stored, the fractions were neutralized with phosphate buffer. (This is important; if the CSF is stored at pH 2.1, in the presence of acetonitrile, almost all of the biological activity will be lost.)

For optimal recovery of CSF, the neutralized microbore fractions should be stored at −20 °C (to prevent the evaporation of the acetonitrile).

Although the degree of purification achieved by these final two reversed-phase procedures was modest it was essential to remove the small contaminating proteins to ensure that the CSF biological activity was associated with these major proteins rather than the impurities. The two forms of CSF detectable in Figs. 5 and 6 needed to be completely separated to study the structure and biology of both forms of CSF. For binding studies with 125I-labeled CSF it is also important that each form is fully biologically active. The leading edge of the CSF protein in Fig. 6 may contain some denatured protein, and it was important to remove this before attempting to prepare 125I-labeled CSF for receptor studies.

Analysis of the Two Forms of L-cell CM CSF—Electrophoretic analysis of these two CSFs indicated that the molecules were quite similar (Fig. 8). There was insufficient protein to detect using Coomassie Blue staining, and neither protein was detectable using silver staining. Approximately 1 μg of each form of CSF was radioiodinated (33) and the proteins detected by autoradiography. Under reducing conditions the first protein appeared to consist of two different subunits (molecular weight 39,000 and 32,000). On reduction the second CSF species yielded a single polypeptide chain (39,000 daltons) (Fig. 8A). In the absence of reducing agent the molecules migrated as broad bands between 58,000 and 77,000 daltons (Fig. 8B). These results are in agreement with the electrophoretic analysis reported for 125I-labeled CSF-1 (34). The isoelectric focusing results with both of these 125I-labeled CSFs yielded identical results (pl approximately 3.5–4.7, Fig. 8C). The 125I-labeled CSF retained biological activity and was suitable for cell surface receptor studies.

These analytical results and the specific biological activity (2 × 10⁹ colonies/mg, Table I) indicated that both molecular forms of L-cell CSF had been completely purified. However,
FIG. 4. Size exclusion chromatography TSK3000 SW. Aliquots (500 µl) of the active concentrate from the Ultrogel AcA44 column were chromatographed on a TSK3000 SW column equilibrated with an aqueous solution of NaCl (0.9%, w/v, pH 5.6) at a flow rate of 1 ml/min. The protein profile (—) was monitored by absorbance at 215 nm and the CSF (■) by the bone marrow colony assay.

FIG. 5. Preparative RP-HPLC. Ultrapore RPSC (300 Å, C3 equilibrated with 0.9% NaCl, in water at pH 2.1) of CSF (previously eluted from the TSK3000 SW column, Fig. 4) using an acetonitrile gradient (0–60% at 1 ml/min) to elute the proteins (—). The CSF (■) eluted toward the end of the gradient, and the peak of biological activity coincided with the final two protein peaks.

our analysis of the type of bone marrow colonies stimulated by both forms of the L-cell CSF molecule (Table II) was significantly different from the previous reports (2, 16). First, both molecules stimulated granulocyte, granulocyte-macrophage, and macrophage colonies and second, the earlier eluting form appeared to stimulate more pure granulocytic colonies (Table II). Three separate preparations of the two forms (A and B) of L-cell CM CSF were prepared, and the distribution of granulocyte, granulocyte-macrophage, and macrophage colonies determined for each preparation (Table II). χ² analysis indicated that the distribution of colony types stimulated by each preparation of the earlier eluting CSF (peak A) was the same. Similarly, the colony distribution types stimulated by the three preparations of the late eluting CSF (peak B) was the same. However, the colony distributions stimulated by the peak A and peak B forms of L-cell CM were significantly different (χ² = 42.0, p < 0.001). All of these comparisons were made using a maximal concentration of the two forms of L-cell CM CSF; however, a similar distribution of colony morphologies was observed at approximately half maximal stimulation (Table II). As distinct from GM-CSF from mouse lung-conditioned medium (1), even at high concentrations, both forms of L-cell CSF stimulated predominantly macrophage colonies.

It is not clear why previous studies found that purified mouse L-cell CSF stimulated only macrophage colonies (2). Perhaps the culture conditions (including the mouse strain from which the bone marrow cells were obtained), the method of selecting colonies for typing, or the staining technique...
Purification of Two Forms of M-CSF from L-cell CM

**Fig. 7.** Microbore RP-HPLC-C8 Brownlee “guard” column. Aliquots (100 µl) of the two pools of fractions containing CSF (A, 50–51 min, Fig. 6 and B, 53–54 min, Fig. 6) were chromatographed separately on the Brownlee Aquapore RP-300 microbore “guard” column. Before the chromatography the CSF sample was diluted with an equal volume of aqueous trifluoroacetic acid (0.15%, v/v, containing Tween 20, 0.02%, v/v) and loaded onto the column. The proteins (—) were eluted with an acetonitrile gradient and monitored at 210 nm. The CSF (■) was detected using the bone marrow colony assay. Fractions (100 µl) were collected in polypropylene tubes. CSF for disks. Fractions to be stored were neutralized by adding 10 µl of a sodium phosphate buffer (0.1 M, pH 7.8, containing Tween 20, 0.2%, v/v) and loaded onto the column. The proteins were blotted onto nitrocellulose. The low pH standard proteins (Pharmacia) were detected by silver staining the nitrocellulose and the 125I-labeled CSFs by autoradiography.

**Fig. 8.** Electrophoretic analysis of the 125I-labeled CSF. The L-cell CSFs did not stain with silver, and it was necessary to assess the purity by radioiodinating the CSF preparations and detecting the iodinated proteins by autoradiography. A, SDS-polyacrylamide gel (10%) electrophoresis after treatment of the samples with dithiothreitol (10 mM); lane a, 125I-labeled CSF-A (from microbore RP-HPLC, Fig. 7A); lane b, 125I-labeled CSF-B (from microbore RP-HPLC, Fig. 7B). The molecular weight standards (Pharmacia) were detected by silver staining the nitrocellulose and the 125I-labeled CSFs by autoradiography. Lane c, 125I-labeled CSF-A; lane d, 125I-labeled CSF-B; lane e, 125I-labeled CSF-B.

**TABLE II**

Morphological analysis of bone marrow colonies stimulated by L-cell CSFs purified using reversed phase HPLC

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of colonies stimulated</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G</td>
</tr>
<tr>
<td><strong>RP-HPLC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak A</td>
<td>98</td>
<td>23</td>
</tr>
<tr>
<td>Peak B</td>
<td>99</td>
<td>5</td>
</tr>
<tr>
<td>Experiment 2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak A</td>
<td>98</td>
<td>16</td>
</tr>
<tr>
<td>Peak B</td>
<td>127</td>
<td>1</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak A</td>
<td>0.1%, v/v^</td>
<td>127</td>
</tr>
<tr>
<td>Peak B</td>
<td>0.02%, v/v^</td>
<td>75</td>
</tr>
<tr>
<td>L-cell CM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(unfractionated)^</td>
<td></td>
<td>97</td>
</tr>
<tr>
<td><em>L-cell CM (unfractionated)</em></td>
<td>124</td>
<td>48</td>
</tr>
</tbody>
</table>

\^ Fractions were assayed at a final concentration of either 0.1%, v/v, and/or 0.02% (Experiment 3) using 75,000 C87BL bone marrow cells in standard semisolid agar cultures (18).

\* Colony morphology was determined after 7 days of incubation by typing 50–100 sequential colonies (3).

\^ Independent reversed-phase HPLC separations performed under identical conditions to those described for Fig. 7.

\* See “Materials and Methods.”

\* Ref. 1.

**TABLE III**

Edman degradation of L-Cell CSFs

<table>
<thead>
<tr>
<th>Position</th>
<th>Phenylthiodyantoin derivative</th>
<th>Peak A yield*</th>
<th>Peak B yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol</td>
<td>pmol</td>
<td>pmol</td>
</tr>
<tr>
<td>1</td>
<td>Lys</td>
<td>118.6</td>
<td>7.0</td>
</tr>
<tr>
<td>2</td>
<td>Glu</td>
<td>12.5</td>
<td>57.5</td>
</tr>
<tr>
<td>3</td>
<td>Val</td>
<td>16.9</td>
<td>72.6</td>
</tr>
<tr>
<td>4</td>
<td>Ser</td>
<td>0.5</td>
<td>4.0</td>
</tr>
<tr>
<td>5</td>
<td>Glu</td>
<td>7.6</td>
<td>30.9</td>
</tr>
<tr>
<td>6</td>
<td>His</td>
<td>0.5</td>
<td>9.4</td>
</tr>
<tr>
<td>7</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Ser</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>His</td>
<td>3.0</td>
<td>10.0</td>
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<tr>
<td>10</td>
<td>Met</td>
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<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>Gly</td>
<td>2.0</td>
<td>12.5</td>
</tr>
<tr>
<td>13</td>
<td>Asn</td>
<td>4.5</td>
<td>11.0</td>
</tr>
</tbody>
</table>

* Yields/degradation cycle of the most prominent amino acid phenylthiodyantoin (R) and the yields of that residue in the preceding (R − 1) and the following cycle (R + 1). biases the results sufficiently to affect the number of granulocytic and granulocyte-macrophage colonies detected. If only the largest colonies (i.e. greater than 500 cells) are scored, both of these stimuli would appear to be macrophage-specific CSFs. The apparent increase in granulocytic colonies with the early eluting form may be partly dependent on concentration; at higher concentrations there appears to be more macrophage colonies. Three different batches of these two CSFs have now been prepared, and even though the earlier eluting form appeared to stimulate more granulocytic colonies, it was clear that both CSFs could stimulate all three colony types.
amino acids of the sequences were identical. All of these residues, except position 7, could be assigned unequivocally. For each protein there was only one amino acid sequence, residues, except position 7, could be assigned unequivocally. This result is not a result of N-terminal or internal proteolysis which would generate several new terminal sequences. Taken together with the data on the glycosidase treatment of L-cell CSF (CSF-1, Ref. 39), these results indicate that the different subunits observed (Fig. 8A, lane a) for the earlier eluting form of the L-cell CSF (Fig. 7A) are glycosylated to different extents.

A search of the Dayhoff protein sequence database failed to locate any proteins with sequence homology to the N-terminal of L-cell CSF. The recent structural data for GM-CSF (44), murine IL-2 (37), murine IL-3 (45), and G-CSF also indicated that there is no close homology with other hemopoietic growth factors. The L-cell CM CSFs are unique hemopoietic growth factors in that there are identical polypeptide chains which need to be associated for biological activity. We are hopeful that the data we have obtained about the N-terminal sequence of L-cell CM CSF will allow the isolation of the corresponding cDNA and thus to a complete sequence analysis. However, the availability of an HPLC system for the purification of the L-cell CSFs will also facilitate the study of the modification and cellular processing, as it is now possible to analyze any of the derivatives or degradation products at high resolution.

**Note Added in Proof**—Since the submission of this article Miller Ben-Avram et al. have reported an N-terminal amino acid sequence for M-CSF isolated from mouse L-cell-conditioned medium (Miller Ben-Avram, C., Shively, J. E., Shadduck, R. K., Waheed, A., Rajavashisth, T., and Lusis, A. J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 4486–4489). Their sequence is similar to the sequence we obtained; however, they report Cys-Cys-Cys at positions 6–8. On careful checking of our data we believe our sequence to be correct and can confirm their other assignments out to position 24.

**REFERENCES**

35. Deleted in proof
40. Deleted in proof
41. Deleted in proof
42. Deleted in proof
43. Deleted in proof
46. Deleted in proof