Purification and Properties of Colony-stimulating Factor from Mouse Lung-conditioned Medium*

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Colony-stimulating factor, which specifically stimulates mouse bone marrow cells to proliferate *in vitro* and generate colonies of granulocytes, or macrophages, or both, was purified 3500-fold from mouse lung-conditioned medium. Analysis by discontinuous polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate indicated that there was a single protein component. All of the colony-stimulating activity was coincident with the protein band. The molecular weight of colony-stimulating factor estimated by gel filtration was approximately 29,000 and by electrophoresis approximately 23,000.

The specific activity of purified colony-stimulating factor from mouse lung-conditioned medium was 7×10^7 colonies/mg of protein. Most of the colony-stimulating factor from mouse lung-conditioned medium bound to concanavalin A-Sepharose, indicating that it is a glycoprotein. The small percentage of colony-stimulating factor in mouse lung-conditioned medium which did not bind to concanavalin A-Sepharose appeared to represent molecules which lacked the carbohydrate moieties required for binding to this lectin. It was necessary to include low concentrations (<0.01%, v/v) of polymers such as gelatin and polyethylene glycol, or nonionic detergents such as Triton X-100, in all of the buffers used throughout the purification scheme, otherwise colony-stimulating factor was lost from solution. At high concentrations (>20 μg/ml) the factor stimulated the formation of granulocytic, macrophage, and mixed colonies from $C_{57}BL$ mouse bone marrow cells. As the concentration of purified colony-stimulating factor was decreased, the frequency of colonies containing granulocytes also decreased. At low concentrations of colony-stimulating factor (<70 pg/ml) only macrophage colonies were stimulated.

The differentiation and proliferation of certain hemopoietic cells has been shown to be dependent on specific glycoproteins (1-3). One of these glycoproteins, colony-stimulating factor, has been shown to be necessary for the proliferation *in vitro* of

specific progenitor cells to form colonies of mature neutrophilic granulocytes, or macrophages, or both. GM-CSF[1] occurs in animal sera (4) and urine (5), as well as in tissue culture media conditioned by a variety of tissues and cell types in culture (6, 7). There appears to be a large variation in the amount of GM-CSF present in these different sources: specific activities range from 75 colonies/mg of protein in human urine (8) and 890 colonies/mg of protein in serum from mice treated with endotoxin (9), to 60,000 colonies/mg of protein in mouse lung conditioned-medium (10, 11). Colony-stimulating activity from MLCM was associated with the α_2-globulin peak on electrophoresis (11), and its apparent sedimentation coefficient (11) indicated that its molecular weight was considerably less than GM-CSF from human urine (8).

EXPERIMENTAL PROCEDURES

Materials – DEAE-cellulose (DE52) was obtained from Whatman, concanavalin A-Sepharose (Lot 5761) from Pharmacia and Ultrogel AcA44 from LKB, Sweden. Sodium dodecyl sulfate was obtained from British Drug Houses, as their "specially" pure grade. Dulbecco's Modified Eagle's medium was purchased from Gibco, fetal calf serum from Laboratory Services, and horse serum from Commonwealth Serum Laboratories. Endotoxin was obtained from Dr. C. Jenkins, University of Adelaide. Deionized distilled water was used throughout these experiments. Chemicals were analytical reagent grade unless otherwise specified. Protein was concentrated using Diaflo UM-2 or PM-10 membranes in an Amicon thin channel ultrafiltration apparatus (TCF 10) or an Amicon stirred micro-ultrafiltration cell (8 MC). Membranes (0.45 μ) for sterilizing solutions by filtration were obtained from Millipore Corp. Na^{125}I was obtained from the Radiochemical Centre, Amersham.

Protein Estimations – Protein was estimated by the method of Lowry *et al.* (12) using bovine serum albumin as a standard. The absorbance at 280 nm was used to monitor the relative protein concentrations of column effluents. Densitometry of Coomassie blue R-250-stained polyacrylamide gels (13) was carried out using a Canalco model J densitometer.

Iodination of Proteins – Biologically active ^{125}I-labeled GM-CSF was prepared by a modification of the chloramine-T method (14, 15). Sodium phosphate buffer (1 μl of a 0.5 M solution, pH 7.0), tracer-free Na^{125} I (5 μl, 78.5 μCi/μl), dimethylsulfoxide (20 μl), and chloramine-T (10 μl of a 10 μg/μl of solution in water) were added to 20 μl of GM-CSF solution (approximately 1 μg in 0.02 M sodium acetate buffer, pH 5.0, 0.01% Triton X-100, and 0.02% sodium azide). After 10 min at 0° the reaction was stopped with 10 μl of sodium metabisulfite (24 μg/μl, in water) and 5 μl of potassium iodide (0.1 M in water). Cytochrome c (5 μl of a 20 μg/μl of solution in water) was added as a

carrier protein and the ^{125}I-labeled protein isolated on a Bio-Gel P6 column (5 × 120 mm).

Storage of GM-CSF — Partially purified GM-CSF (Step 4) was diluted to a protein concentration of 2 µg/ml in 0.03 M Tris/HCl buffer, pH 7.4, containing the various compounds listed in Table I, and after standing for 18 h at 4°, 0.1-ml aliquots from each incubation were assayed for GM-CSF activity (see below). In order to retain the biological activity from GM-CSF on storage it was necessary to include Triton X-100 (0.01%, v/v) in all of the buffers.

Polyacrylamide Gel Electrophoresis — The system used for SDS polyacrylamide gel electrophoresis was based on the stacking gel system described by Laemmli (16). The protein sample was incubated at 37° for 30 min in the presence of SDS (1%, w/v) and 2-mercaptoethanol (5%, w/v), and then applied to the upper surface of the stacking gel. The stacking gel was made with 3% acrylamide monomer and the initial acrylamide monomer concentration in the separating gel was varied between 8 and 15%. The ratio of bisacrylamide to acrylamide was maintained at 1:30. The stacking gel was polymerized in the presence of riboflavin (17) and the running gel by using tetramethylethylenediamine (0.25 µl/ml) and ammonium persulfate (0.3 mg/ml). The time taken for polymerization was usually between 30 and 60 min. Gels were stained in 0.1% Coomassie blue in 50% methanol, 10% acetic acid, 40% water, and destained for 4 h in 10% trichloroacetic acid, 7% acetic acid, followed by overnight destaining in 7% acetic acid. GM-CSF was located after electrophoresis by freezing the gel in a mixture of alcohol and dry ice, slicing horizontally (2.5-mm intervals) and crushing the slices into 1 ml of distilled water containing fetal calf serum (5%, v/v). Dowex mixed bed ion exchange resin AG 501-X8 (D) (0.1 g) was added to the eluate and the gel extracted for 24 h at 4°. The polyacrylamide gel and ion exchange particles were removed from the eluate by Millipore filtration (0.45 µ).

Preparative discontinuous polyacrylamide gel electrophoresis (17, 18) in the absence of SDS was performed using gel tubes (1.5 × 10 cm) with a 3-ml stacking gel polymerized with riboflavin (17) above a 7-ml acrylamide (15%), bisacrylamide (0.5%) running gel. The running gel was polymerized with tetramethylethylenediamine (0.25 µl/ml) and ammonium persulfate (0.3 mg/ml). The upper and lower reservoir buffers were made with 0.025 M Tris/glycine, pH 8.3, the running buffer with 0.035 M Tris/HCl, pH 8.8, and the spacer gel buffer with 0.063 M Tris/HCl, pH 6.8.

Biological Assay for GM-CSF Activity — Samples to be assayed for GM-CSF activity were diluted with 5% fetal calf serum in distilled water prior to sterilization by filtration through a 0.45 µ Millipore membrane. The presence of fetal calf serum reduces the loss of GM-CSF activity due to adsorption on the membranes.[2] Aliquots (0.1 ml) of each sample dilution were assayed by the method of Metcalf (19), excepting that Dulbecco's modified Eagle's medium containing fetal calf serum (15%) and horse serum (5%) was used instead of Eagle's minimal essential medium. A titration of each sample was made using 5-fold serial dilutions and the GM-CSF activity was defined as the calculated number of colonies stimulated by 0.1 ml of the sample as determined from that portion of the titration curve stimulating between 20 to 100 colonies (each colony contained 50 or more cells) in the 1-ml cultures of 75,000 viable C_{57}BL bone marrow cells in 0.3% agar. Under these culture conditions a maximally stimulated culture contains approximately 250 colonies/75,000 mouse bone marrow cells and a culture without GM-CSF has no colonies.

Preparation of Mouse Lung-conditioned Medium (10, 11) — Endotoxin from *Salmonella typhimurium* (0.2 ml of a 25 µg/ml of solution in normal saline) was injected intravenously into each of 8 × 100 adult C_{57}BL mice. (In a day it was convenient for one person to prepare MLCM from one hundred mice.) Three hours after injection the mice were killed and each pair of lungs was removed, rinsed in Eisen's balanced salt solution (20), and then transferred into 5 ml of serum-free Dulbecco's modified Eagle's medium in sterile Falcon tubes (17 × 100 mm). After incubation at 37°C for 48 h in a humidified 10% CO_2 incubator, the mouse lung-conditioned medium was filtered through a double layer of cotton gauze and stored at −15°.

RESULTS AND DISCUSSION

Loss of Colony-stimulating Activity on Storage

During the initial attempts to purify GM-CSF most of the colony-stimulating activity was lost when the preparation was stored for several days. GM-CSF from Step 4 of the purification scheme was used to search for compounds which would minimize the loss of colony-stimulating activity on storage.

Addition of proteins such as bovine serum albumin and gelatin, and carbohydrate polymers such as Ficoll, polyethylene glycol, and methylcellulose prevented the loss of GM-CSF activity on storage (Table I). Low concentrations of nonionic detergents such as Tween 20, Tween 80, and Triton X-100 also prevented losses of GM-CSF activity (Table I). Low molecular weight carbohydrates such as glycerol, mannitol, and sucrose did not prevent the loss of CSF activity. It is likely that the nonionic detergents prevented the loss of colony-stimulating activity by reducing the nonspecific adsorption of GM-CSF to glass and plastic surfaces.

Purification of GM-CSF

The following procedures were performed below 8° and buffers contained 0.02% sodium azide and 0.01% Triton X-100 (unless otherwise stated). pH values were measured against Radiometer standard phosphate buffer, pH 6.5 at 8°.

Step 1 — Mouse lung-conditioned medium was heated at 56° for 30 min and all insoluble material removed by centrifugation (12,000 × g for 15 min). The supernatant fluid was dialyzed against 3 × 10 volumes of deionized distilled water and the resulting precipitate removed by centrifugation (12,000 × g for 15 min). The supernatant fluid was retained as Step 1 GM-CSF and this had a specific activity of 2×10^4 colonies/mg of protein (Table II).

Step 2 — Calcium phosphate gel (21) was stirred into the solution of GM-CSF from Step 1 (1 ml of standard gel/10 mg of protein). After 2 h most of the supernatant fluid was decanted and the calcium phosphate gel collected by centrifugation (10,000 × g for 20 min). The gel was washed initially with 2 volumes of 0.01 M sodium phosphate, pH 6.8, and then with 1.5 volumes of 0.10 M sodium phosphate buffer, pH 6.8, to elute GM-CSF. The eluate containing GM-CSF was dialyzed against 3 × 10 volumes of 0.06 M Tris/HCl buffer, pH 7.4.

Step 3 — GM-CSF from Step 2 was chromatographed on a column of DEAE-cellulose (2.3 × 75 cm) equilibrated with 0.06 M Tris/HCl buffer, pH 7.4. A flow rate of 60 ml/h was maintained during the chromatography and 10-ml fractions were collected. After the absorbance at 280 nm returned to the baseline, a linear gradient of sodium chloride (0.2 mM/ml) was

TABLE I

Effect of different compounds on storage of GM-CSF from MLCM

GM-CSF (2 µg/ml in 0.03 M Tris/HCl, pH 7.4, Step 4) was stored for 18 h at 4° in the presence of the compounds listed below. Aliquots were assayed for GM-CSF and the results expressed as a percentage of the number of colonies stimulated by the GM-CSF sample stabilized by bovine serum albumin (which produced 37 colonies).

Compound	Concentration in CSF solution	Relative no. of colonies ± S.D.
	%	
None		5.4 ± 2.7
Bovine serum albumin	0.5	100 ± 19
Gelatin	0.5	100 ± 17
Glycerol	25	5.4 ± 2.7
Sucrose	30	10.8 ± 2.7
Mannitol	15	10.8 ± 2.7
Ficoll	15	67 ± 10
Polyethylene glycol	25	95 ± 19
Methylcellulose	2.5	100 ± 14
Tween 20	0.01	98 ± 15
Tween 80	0.01	95 ± 11
Triton X-100	0.01	95 ± 16

[2] J. Camakaris, and J. W. Sheridan, unpublished results.

TABLE II

Purification of GM-CSF from mouse lung-conditioned medium

Purification step	Volume	Total activity (× 10⁻⁶)	Total protein	Specific activity (× 10⁻⁶)	Purification	Yield
	ml	*colonies*	*mg*	*colonies/mg*	*-fold*	*%*
1. MLCM	3600	103	4800	0.02	1	100
2. Calcium phosphate gel	42	90	620	0.14	2	87
3. DEAE-cellulose	17	54	60	0.9	45	52
4. Concanavalin A-Sepharose	22	26	13	2.0	100	25
5. Ultrogel AcA44	7	24	0.7	34	2000	23
6. Preparative gel electrophoresis	2	8	0.12	70	3500	8

applied. Fractions containing GM-CSF (which eluted between 100 and 400 ml after the start of the gradient, Fig. 1) were pooled, concentrated by ultrafiltration over a Diaflo PM-10 membrane (to about 20 ml), and dialyzed against 2 × 50 volumes of 0.2 M sodium acetate buffer, pH 5.0, which contained Ca^{2+}, Mg^{2+}, and Mn^{2+} ions (1 mM). The peak of colony-stimulating activity eluted from the DEAE-cellulose column at 0.05 M NaCl (Fig. 1) and the specific activity (900,000 colonies/mg) represented a 45-fold purification over the specific activity of GM-CSF in the initial MLCM.

Step 4 — GM-CSF from Step 3 was applied to a column containing concanavalin A-Sepharose (1.5 × 25 cm) which had been equilibrated with 0.2 M sodium acetate buffer, pH 5.0, containing Ca^{2+}, Mn^{2+}, and Mg^{2+} ions (1 mM). The column flow rate was maintained at 20 ml/h and 4-ml fractions were collected. Above pH 5.5 considerable quantities of concanavalin A (10 to 20 μg/ml) contaminated the buffer eluting from the concanavalin A-Sepharose column. At pH 5.0, in acetate buffer, the concentration of concanavalin A in the column eluate was less than 10 ng/ml. After the first peak of protein eluted from the column, a further 130 ml of starting buffer was applied and the bound protein eluted with the same buffer containing 0.05 M methyl-α-D-glucopyranoside (Fig. 2). Two peaks of GM-CSF (Fig. 2, *A* and *B*) eluted from the concanavalin A-Sepharose column and these were both coincident with the protein peaks. The fractions containing GM-CSF were pooled separately and each pool was concentrated to approximately 20 ml. GM-CSF which eluted with 0.05 M methyl-α-D-glucopyranoside (*Pool B*) (contained 80% of the total biological activity recovered from the concanavalin A-Sepharose column. The GM-CSF contained in Pool B was dialyzed against 3 × 10 volumes of Tris/HCl buffer (0.10 M, pH 7.4) to yield GM-CSF which had a specific activity of 2,000,000 colonies/mg of protein.

The fact that some of the GM-CSF did not bind to the concanavalin A-Sepharose column (*Pool A*, Fig. 2) was not due to insufficient binding capacity of the column. The application of much smaller amounts of GM-CSF (from Step 3) to this concanavalin A-Sepharose column led to a colony-stimulating activity profile similar to that shown in Fig. 2. Previous studies have shown that all of the colony-stimulating activity in human urine binds to concanavalin A-Sepharose and that it can be eluted with 0.1 M methyl-α-D-glucopyranoside (8, 22, 23). Two possible explanations for the appearance of GM-CSF in the column void volume are: (*a*) that there were at least two different molecules with colony-stimulating activity present in the material from Step 3 of the purification, one or more of which did not have the α-linked mannose or glucose terminal carbohydrate moieties required for interacting with concanavalin A (24); or (*b*) that GM-CSF binds both to free concanavalin A and to concanavalin A-Sepharose and there is sufficient free concanavalin A leaking off the affinity column to carry some of the CSF activity through the column before it
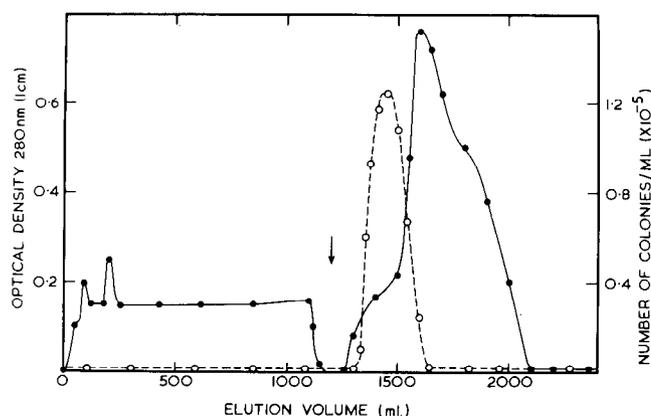

FIG. 1. DEAE-cellulose elution profile (Step 3). The column dimensions were 2.3 × 75 cm. ●——●, protein; ○- - -○, colony-stimulating activity.
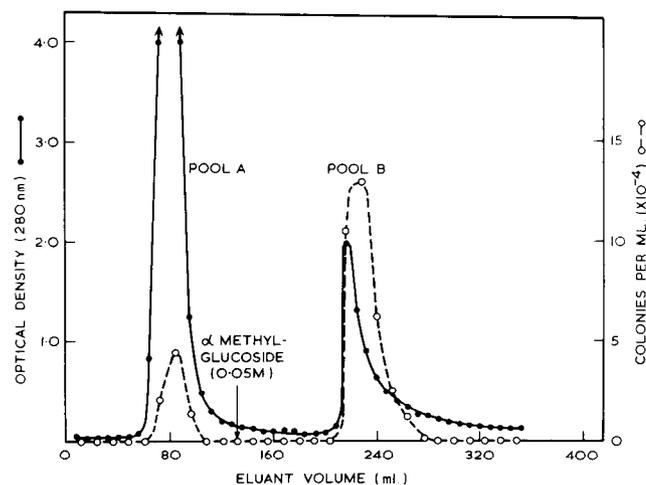

FIG. 2. Concanavalin A-Sepharose elution profile (Step 4). The column dimensions were 1.5 × 25 cm. ●——●, protein; ○- - -○, colony-stimulating activity.

interacts with the concanavalin A-Sepharose. These two possibilities were tested by rechromatography of Pools A and B on concanavalin A-Sepharose. As expected, all of the Pool A GM-CSF eluted at the void volume and all of GM-CSF in Pool B bound to the column. This suggested that some of the GM-CSF molecules in the initial conditioned medium were lacking carbohydrate moieties required for binding to concanavalin A (24). It was possible that the GM-CSF in Pool A was still bound to free concanavalin A and that this complex did not bind to concanavalin A-Sepharose. The elution volume of GM-CSF on gel filtration from Pools A and B of the concanavalin A-Sepharose column were the same, which indicated that the GM-CSF in Pool A was not formed in a complex with another

protein (25). Incomplete addition of carbohydrate moieties to other glycoproteins, synthesized *in vitro,* has been reported previously (26).

Step 5 — GM-CSF from Step 4 was applied to a column of Ultrogel AcA44 (1.7 × 200 cm) equilibrated with 0.10 M Tris/HCl buffer, pH 7.4. The flow rate was maintained at 20 ml/h and 8-ml fractions were collected. GM-CSF eluted from the column between 600 and 700 ml (Fig. 3); these fractions were pooled and concentrated to 7 ml using a Diaflo UM-2 ultrafiltration membrane. Several proteins of known molecular weight (bovine serum albumin, ovalbumin, concanavalin A, α-chymotrypsinogen, and myoglobin) were also chromatographed on this column and the linear relationship between the logarithm of the molecular weight of a protein and its fractional elution volume (25) used to estimate the apparent molecular weight of GM-CSF (29,000).

Step 6 — Sucrose (10%, w/v) and bromphenol blue (10 μl of a 1% w/v in distilled water) were added to Step 5 GM-CSF and the solution (7 ml) applied to the preparative gel electrophoresis column. Until the dye had completely entered the stacking gel (approximately 2 h) the electrophoresis was run at a constant voltage (50 V). After the dye marker had stacked, the voltage was increased (100 V, the current was approximately 8 mA) and the electrophoresis run for twice the time taken for the bromphenol blue marker to run through the gel. At the completion of the electrophoresis, the gel was sliced at 1.5-mm intervals and eluted into aliquots of 0.06 M Tris/HCl buffer, pH 7.4 (2 ml). Fractions containing GM-CSF were pooled and concentrated 4-fold on a Diaflo UM-2 ultrafiltration membrane. Preparative polyacrylamide gel electrophoresis yielded approximately 120 μg of GM-CSF with a specific activity of 7 × 10^7 colonies/mg, which represented a 3500-fold purification with respect to the initial mouse lung-conditioned medium. The results of each step in the purification scheme have been summarized in Table II.

Molecular Weight Studies on CSF

SDS is highly toxic to bone marrow cells from C_{57}BL mice, so that no colonies can be stimulated by GM-CSF when the SDS concentration in the culture dish is above 7 μM. Thus it was not possible to assay for GM-CSF in untreated eluates from polyacrylamide gel slices after electrophoresis in the presence of SDS. Fortunately the SDS could be removed using a mixed bed ion exchange resin (see "Experimental Procedures"), allowing a quantitative estimate of the molecular weight of GM-CSF under strongly dissociating conditions. A least squares line of regression, for the relationship between the logarithms of the molecular weights of a set of standard proteins and their
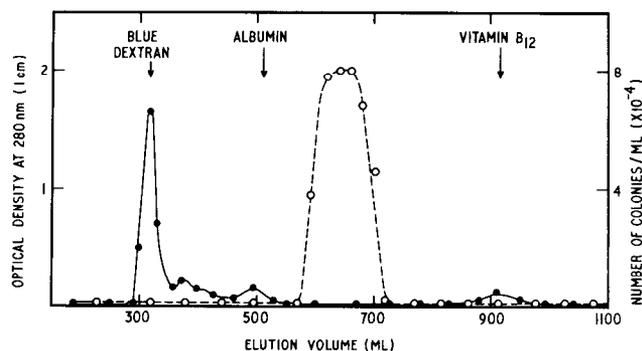
relative electrophoretic mobility (Fig. 4), was used to estimate the apparent molecular weight of GM-CSF from MLCM. The value obtained was 23,000. Similar experiments using higher concentrations of acrylamide (12 and 15%) yielded an apparent molecular weight between 23,000 and 25,000. This agreed with earlier results where GM-CSF from Step 1 (calcium phosphate gel absorption) was used for zone sedimentation studies under nondissociating conditions (11). The apparent molecular weight of GM-CSF from the gel filtration studies was approximately 29,000 (Fig. 3), so it appears that GM-CSF from MLCM normally exists as a monomeric protein. This slightly higher value for the apparent molecular weight from the gel filtration experiment could be attributed to the large hydration sphere associated with carbohydrate moieties (25).

Criteria of Purity

Analysis of GM-CSF from the final step in the purification by polyacrylamide gel electrophoresis yielded a single band of protein which contained all of the biological activity. On 10% and 12% polyacrylamide gels (17, 18) GM-CSF electrophoresed as a broad band typical of many glycoproteins (27). When 15% polyacrylamide gels were used, however, GM-CSF stacked to form a single sharp protein band which had an electrophoretic mobility of 0.27 relative to bromphenol blue. A typical protein activity profile for this electrophoretograph is shown in Fig. 5. Within the accuracy of the bioassay and protein determinations the protein and activity peaks appear to be coincident. GM-CSF was not easily detected on polyacrylamide gels using Coomassie blue R-250 to stain for protein. There did not appear to be an unusual amount of ^{125}I-labeled GM-CSF leached from the gels during the destaining in acetic acid (7%, v/v), so it was probable that the dye did not bind tightly to the protein. The visualization of GM-CSF with Coomassie blue was improved when the initial destaining was performed in the presence of trichloroacetic acid (10%, v/v) for 4 h before using acetic acid (7%, v/v). When the gel was left in contact with trichloroacetic acid overnight the protein bands faded. Polyacrylamide gel electrophoresis, of GM-CSF from the final step in the presence of SDS also indicated that the preparation was homogeneous. The protein and activity profiles of pure GM-CSF from MLCM on a 15% polyacrylamide gel in the presence of

FIG. 3. Gel filtration (Ultrogel AcA44) elution profile (Step 5). The column dimensions were 1.7 × 200 cm. ●———●, protein; ○- - -○, colony-stimulating activity.
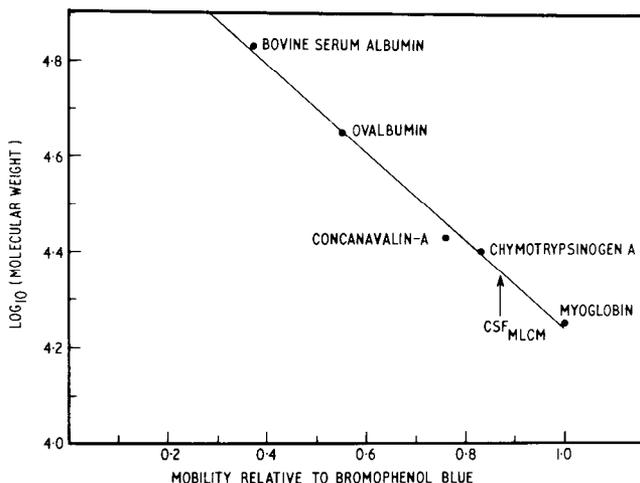

FIG. 4. Molecular weight determination of GM-CSF by SDS electrophoresis (9.6% polyacrylamide gels). The molecular weights assumed for the marker proteins were: bovine serum albumin, 67,000; ovalbumin 45,000; concanavalin A, 27,000; α-chymotrypsinogen, 27,000; myoglobin, 17,800. The least squares line of regression was used to predict the molecular weight of GM-CSF (23,000).

SDS are shown in Fig. 5, again both peaks were coincident (and their mobility relative to bromphenol blue was 0.52). ^{125}I-labeled GM-CSF from MLCM also showed a single γ irradiation peak which co-electrophoresed with the protein and biological activity peaks shown in Fig. 5.

Morphology of Colonies Stimulated by GM-CSF from MLCM

When unpurified GM-CSF from MLCM is used to stimulate colony formation by mouse bone marrow cells, pure granulocyte, pure macrophage, or mixed granulocyte and macrophage colonies develop (11, 28). In assay cultures stimulated by material from purification Steps 1 to 6, no significant differences were observed in the relative frequencies of these major types of colonies (Table III). The slightly lower percentages of granulocytic colonies in cultures stimulated by GM-CSF from Step 4 (concanavalin A-Sepharose chromatography) and Step 6 (polyacrylamide gel electrophoresis) in the purification were not significantly different and were a consequence of using slightly lower GM-CSF concentrations than in the cultures for the other steps of the purification (11).
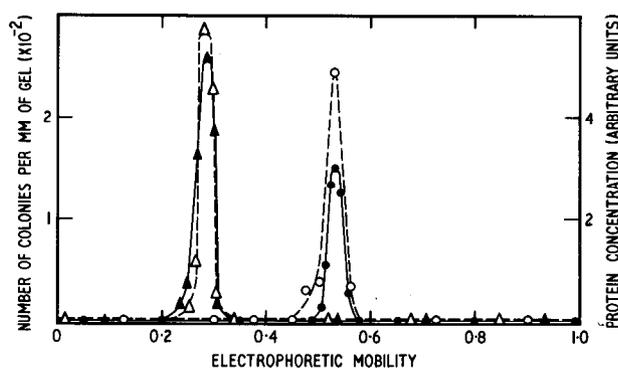

FIG. 5. Protein and colony-stimulating profiles of purified GM-CSF from MLCM (Step 6) on discontinuous polyacrylamide gel (15%) electrophoresis in the presence and absence of sodium dodecyl sulfate. Half of each gel was stained with Coomassie blue R-250 and the other half was sliced and bioassayed for GM-CSF (19). In the absence of sodium dodecyl sulfate: ▲——▲, protein; △- - -△, colony-stimulating activity. In the presence of sodium dodecyl sulfate: ●——●, protein; ○- - -○, colony-stimulating activity.

TABLE III

Colony morphology in cultures of $C_{57}BL$ mouse bone marrow cells stimulated by CSF from MLCM at various stages of purification

Each 1-ml culture contained 75,000 $C_{57}BL$ mouse bone marrow cells in 0.3% agar and was stimulated by 0.1 ml of the CSF preparation. Colony counts were made after 7 days of incubation at 37° in a fully humidified atmosphere containing 10% CO_2 (19). Approximately 40 discrete colonies were removed from each type of culture and classified as granulocytic (greater than 98% granulocytes), macrophage (greater than 98% macrophage), or mixed (a colony containing more than 2% of both cell types) as determined from staining with 0.6% orcein in 60% acetic acid (9, 28).

Purification step	No. of colonies	Mean percentage of colonies		
		Granu-locyte	Mixed	Macro-phage
1. MLCM	132	27	40	33
2. Calcium phosphate gel	129	60	27	13
3. DEAE-cellulose	104	37	26	37
4. Concanavalin A-Sepharose (Pool B)	33	12	28	60
5. Ultrogel AcA44	64	37	30	33
6. Preparative gel electrophoresis	37	23	12	65

The capacity of apparently homogeneous GM-CSF to stimulate the proliferation of both granulocytic and macrophage populations needs emphasis. Both populations are generated by common progenitor (colony-forming) cells, but the colony-forming cells have been shown to be heterogeneous (29). Some colony-forming cells have a predetermined capacity to form only granulocytes, while others form mixed colonies which ultimately transform to macrophage colonies. The present results with purified GM-CSF indicate either that this form of CSF can stimulate both types of colony-forming cells and their progeny, or less likely, that two or more different types of CSF, one specific for granulocytes, the other for macrophages, are present in the apparently homogeneous preparation of GM-CSF from the final step in the purification scheme.

The morphological distribution of colonies stimulated by GM-CSF from the protein which failed to bind to concanavalin A-Sepharose was similar to that produced by GM-CSF from the other purification steps (Table III). This indicated that the carbohydrate moieties on GM-CSF which interact with concanavalin A did not determine the differentiative response of the cells on which it acts. GM-CSF eluted from concanavalin A-Sepharose with methyl-α-D-glucopyranoside had no stimulatory activity in the assay for fibroblast growth-enhancing protein (30) or in the assay for somatomedin sulfation factor (31).

The purification scheme described in this report led to GM-CSF with a specific activity of approximately 7×10^7 colonies/mg of protein (*i.e.* 14 pg of GM-CSF was required to stimulate a single colony). This represented a 3500-fold purification compared to the starting material, a 100,000-fold increase in specific activity compared to whole endotoxin serum (9), and a 1.3 $\times 10^6$-fold increase over the activity of CSF in unfractionated human urine (8). It was reported previously (10) that the specific activity of GM-CSF from MLCM was approximately 10^{10} colonies/mg. This value was derived from an analysis of a gradient polyacrylamide gel electrophoretogram of partially purified GM-CSF using Coomassie blue to determine protein concentration (10), but GM-CSF from MLCM does not stain well with Coomassie blue R-250. That original estimate of the specific activity (10) of GM-CSF from MLCM is unlikely to be correct. A report describing the purification of GM-CSF from mouse L cell-conditioned medium (32) estimated the specific activity of that GM-CSF to be 2×10^6 colonies/mg of protein (32), which is considerably less than the value found here for GM-CSF from MLCM and the value reported for GM-CSF from human urine (8). Recently the purification of mouse L cell-conditioned medium GM-CSF was continued by Stanley et al. (33) and their pure preparation of GM-CSF had a specific activity of 3×10^8 colonies/mg of protein. Both GM-CSF from MLCM and GM-CSF from human urine appear to be glycoproteins. Although the original report on GM-CSF from mouse L cell-conditioned medium (32) indicated that there was no detectable carbohydrate (32), more recently it has been shown that mouse L cell GM-CSF binds to concanavalin A-Sepharose, suggesting that it too is a glycoprotein (33).

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