Granulocyte/macrophage colony-stimulating factor from mouse lung conditioned medium

Purification of multiple forms and radioiodination

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Four forms of mouse granulocyte/macrophage colony-stimulating factor (GM-CSF) were purified 100 000-fold from mouse lung conditioned medium. Each of the CSF species stimulated the formation of both granulocyte and macrophage colonies, and half-maximal stimulation in the semi-solid mouse bone-marrow colony assay occurred at 1 pM. The four GM-CSF species exhibited similar charge micro-heterogeneity, focusing between pH 4.2 and pH 5.2. On SDS/polyacrylamide gels two of the GM-CSF sub-species had apparent M_r values of 23 000, and the other two, 21 000. Treatment with neuraminidase decreased the M_r values of these two sets to 21 000 and 19 000 respectively. Incubation with endoglucosidase F decreased the charge heterogeneity and the M_r of all species to 16 500. A gas-phase radioiodination procedure was used to incorporate 2–3 atoms of ^{125}I /molecule into purified GM-CSF without any loss of biological activity. The ^{125}I -labelled GM-CSF was analysed on a microbore reversed-phase h.p.l.c. column to determine its specific radioactivity directly. This ^{125}I -labelled GM-CSF molecule is suitable for cell-surface receptor-binding studies.

INTRODUCTION

The colony-stimulating factors (CSFs) are a set of glycoproteins responsible for the control of blood-cell production (Dexter, 1984). The initial purification of two of these proteins, i.e. granulocyte/macrophage (GM)-CSF (Burgess *et al.*, 1977) and macrophage (M)-CSF (Stanley & Heard, 1977; also called CSF-1), was achieved by conventional chromatography and polyacrylamide-gel electrophoresis. However, the discovery of several new CSFs, e.g. multi-CSF (Burgess *et al.*, 1980) and granulocyte (G)-CSF (Nicola *et al.*, 1983), and the availability of h.p.l.c. techniques suitable for the purification and high resolution and recovery of protein mixtures (Nice *et al.*, 1979; Cooke *et al.*, 1983), has necessitated the development of an improved method for the purification of GM-CSF. More sensitive analytical techniques (Morrissey 1981; Merrill *et al.*, 1982) have permitted a better assessment of the purification scheme, so that the protein could be obtained in a form suitable for micro-sequence analysis (Simpson & Nice, 1984) and radiolabelling for receptor studies (Guilbert & Stanley, 1980; Nicola & Metcalf, 1984).

The observation of a factor in endotoxin serum, which stimulated leukaemic-cell differentiation (Metcalf, 1980; Lotem *et al.*, 1980; Burgess & Metcalf, 1980), and which appeared to be associated with a G-CSF, initiated a search for a suitable source for this protein (Nicola & Metcalf, 1981). Mouse lung conditioned medium (MLCM) appeared to be the most suitable starting material for the purification of both GM- and G-CSF. This required a change in the initial purification steps for

GM-CS (Burgess *et al.*, 1977), so that both GM-CSF and G-CSF (Nicola *et al.*, 1983) could be recovered in good yield.

The initial steps used to extract the G-CSF did not remove a significant amount of protein from the GM-CSF, but resulted in a high-salt solution, necessitating a different approach to the initial purification steps (Burgess *et al.*, 1977). This purification scheme concentrates on the GM-CSF glycoprotein, which binds to concanavalin A-Sepharose, as it has been difficult to remove the large amount of contaminating protein from the conditioned medium by precipitation or absorption techniques, without co-precipitating the GM-CSF. However, the combination of conventional chromatography, to decrease the total protein mass, followed by several high-resolution h.p.l.c. steps produced four species of GM-CSF in good yield. The biological properties, the extent of glycosylation of the different forms and the radioiodination of the purified GM-CSFs are described in detail. The specific radioactivity of the radioiodinated derivative was determined by reverse-phase h.p.l.c. on a microbore column (Nice *et al.*, 1984). The ^{125}I -labelled GM-CSF retained full biological activity and appears to be suitable for detailed receptor analysis (Walker & Burgess, 1985).

MATERIALS AND METHODS

Chemicals

All reagents were AnalaR or h.p.l.c. grade unless specifically stated. Buffers were prepared in water

Abbreviations used: CSF, colony-stimulating factor; G-CSF, M-CSF, GM-CSF, granulocyte CSF, macrophage CSF, granulocyte/macrophage CSF; MLCM, mouse lung conditioned medium; HFBA, heptafluorobutyric acid.

obtained from a reverse-osmosis Super Q water polishing system (Millipore). All buffers contained NaN_3 (0.02%, w/v) and Tween 20 (0.02%, v/v) unless otherwise stated.

Biological assays for GM-CSF activity

Samples to be assayed for GM-CSF activity were diluted and filter-sterilized, before assay with C57B1 mouse bone-marrow cells as described previously (Metcalf, 1977). [Fractions containing toxic buffers such as acetonitrile were always diluted at least 1:100 before the assay.] Samples containing SDS were treated with a Dowex mixed-bed ion-exchange resin, AG501-X8(D), before sterilization with 0.45 μm Millipore filters.

Preparation of mouse lung conditioned medium (Step 1)

Conditioned medium was prepared in the presence of LiCl from the lungs of endotoxin-treated mice (C57BL/6f/J/WEHI strain, 3–4-month-old males and females) as described previously (Burgess *et al.*, 1977). Initially, the G-CSF was removed by salting out and phenyl-Sepharose chromatography (Nicola *et al.*, 1983).

Chromatographic procedures

Step 2a: salting-out chromatography. Batches (3 litres) of MLCM were concentrated to 100 ml and exchanged into sodium phosphate buffer (0.1 M, pH 6.0) by using a H1 P10 hollow-fibre cartridge in an Amicon DC2A concentrator. Sepharose CL-6B (Pharmacia; 200 ml) was equilibrated with sodium phosphate buffer (0.1 M; pH 6.0) containing $(\text{NH}_4)_2\text{SO}_4$ (1 M), and the packed gel was added to the concentrated MLCM. Then 600 ml of sodium phosphate buffer (0.1 M, pH 6.0) containing $(\text{NH}_4)_2\text{SO}_4$ (4 M) was added slowly (50 ml/h) with continuous mixing. This was done with an overhead stirrer, to avoid damage to the Sepharose beads. A considerable portion of the protein precipitates on to the surface of the Sepharose beads. The Sepharose was washed with sodium phosphate buffer (0.1 M; pH 6.0) containing $(\text{NH}_4)_2\text{SO}_4$ (2.75 M), on a sintered-glass funnel. The washed Sepharose was poured into a column (2.6 cm $\times$ 40 cm), and the proteins were eluted with a linear gradient (600 ml) from 2.75 M- $(\text{NH}_4)_2\text{SO}_4$ in buffer to buffer alone at a flow rate of 25 ml/h, 4.5 ml fractions being collected. The GM-CSF was eluted between 2.5 M- and 1.8 M- $(\text{NH}_4)_2\text{SO}_4$ (Nicola *et al.*, 1983), together with most of the protein, but substantially free of G-CSF (Nicola *et al.*, 1983). To avoid G-CSF contamination, those fractions that contained both GM-CSF and G-CSF were pooled with the G-CSF fractions and further purified by phenyl-Sepharose chromatography (Nicola *et al.*, 1983), i.e. Step 2b below. The fractions containing GM-CSF but not G-CSF were pooled (approximately 90 ml) for the next purification procedure, concanavalin A-Sepharose chromatography.

Step 2b: phenyl-Sepharose CL-6B chromatography. The fractions from the salting-out chromatography which still contained G-CSF were pooled (approx. 110 ml) and applied directly to a column (2.6 cm $\times$ 20 cm) of phenyl-Sepharose CL-6B (Pharmacia) previously equilibrated with sodium phosphate buffer (0.1 M; pH 6.0) containing $(\text{NH}_4)_2\text{SO}_4$ (1 M). The column was washed with 50 ml of this equilibrated buffer, and then the proteins were eluted with a linear gradient (200 ml), starting with the equilibration buffer and finishing with

water containing NaN_3 (0.02%) and Tween 20 (0.02%). The GM-CSF was eluted from the phenyl-Sepharose between 0.8 M- and 0.5 M- $(\text{NH}_4)_2\text{SO}_4$ and contained no G-CSF. The G-CSF was eluted from the phenyl-Sepharose with a second linear gradient (200 ml) from water to ethylene glycol/water (3:2, v/v) containing NaN_3 (0.02%) and Tween 20 (0.02%) (Nicola *et al.*, 1983). The fractions containing GM-CSF were pooled (approx. 50 ml) and combined with the GM-CSF pool from the salting-out chromatography.

Step 3: concanavalin A-Sepharose chromatography. The fractions containing GM-CSF from six salting-out columns and six phenyl-Sepharose columns were pooled, adjusted to pH 5.0 and made 0.2 M with sodium acetate buffer (1 M stock solution) and 1 mM- MgCl_2 (Burgess *et al.*, 1977). Affinity chromatography on concanavalin A-Sepharose was performed as described previously (Burgess *et al.*, 1977), except that 1 ml of packed concanavalin A-Sepharose (Pharmacia) was used for each 50 mg of total protein, to absorb the glycoproteins. After loading the sample, the column was washed with 2 column vol. of buffer before elution of the glycoproteins with a mixture of α -methyl glucopyranoside (0.05 M) and α -methyl mannopyranoside (0.05 M) at a flow rate of 20 ml/h. Retained GM-CSF was recovered in a volume of approx. 80 ml.

Step 4: gel filtration on Ultrogel AcA54. The pool of GM-CSF that bound to the concanavalin A-Sepharose column was concentrated to 10 ml over an Amicon PM-10 membrane and applied to a column (2.4 cm $\times$ 180 cm) of Ultrogel AcA54 equilibrated with Tris/HCl (20 mM; pH 7.4). The flow rate was maintained at 30 ml/h, and 10 ml fractions were collected. The fractions containing GM-CSF were pooled for the final h.p.l.c. (Fig. 1).

H.p.l.c. separations

Preparative separations were performed on a Beckman model 324-40 h.p.l.c. apparatus fitted with a 2 ml injection loop. Large-volume samples were loaded either by multiple injection (less than 10 ml) or by pumping directly through the primary pump (greater than 10 ml). Detection was by sequential u.v. adsorption at 280, 215 or 210 nm (LDC Spectromonitor III) and endogenous fluorescence (Kratos FS950; 214 nm excitation; 340 nm emission). Fractions were recovered with a Pharmacia FRAC100 collector controlled by the Beckman 324-40 microprocessor. Polyethylene glycol (0.005%, w/v) or Tween 20 (0.02%) was added to recovered fractions to minimize loss of protein by non-specific adsorption to the polypropylene collection tubes. Chromatographic conditions for the specific separations are listed below. All separations were achieved at ambient temperature.

Step 5: reversed-phase chromatography (RP-h.p.l.c.) with heptafluorobutyric acid. The pooled fractions containing the GM-CSF from the Ultrogel AcA54 column (in Tris/HCl, 20 mM) were trace-enriched on to a Beckman Ultrapore RPSC column (7.5 cm $\times$ 4.6 mm internal diam.; 5 μm particles, 30 nm pore-size silica chemically bonded with a C_8 alkyl chain) (Nice *et al.*, 1979; Cooke *et al.*, 1983). After equilibration of the

column with starting buffer (water containing HFBA, 0.2%, v/v), retained proteins were eluted with a gradient of acetonitrile [0–50% (v/v), increasing at 1%/min] at a flow rate of 1 ml/min (Fig. 2).

Step 6: anion-exchange h.p.l.c. The pooled fractions from the reversed-phase h.p.l.c. in the presence of HFBA were pooled (5 ml), diluted 10:1 with Tris/HCl (20 mM, pH 8.0) and loaded directly on to a Pharmacia Mono Q column (Burgess *et al.*, 1983) equilibrated with the Tris/HCl buffer. After loading the sample, the proteins were eluted at a flow rate of 1 ml/min with a discontinuous salt (NaCl) gradient in the buffer: 0–0.1 M in 5 min, followed by 0.1–0.15 M in 30 min (Fig. 3).

Step 7: Reversed-phase h.p.l.c. with NaCl and acetonitrile. The presence of HFBA selectively alters the elution characteristics of many proteins by ion-pairing (Bennett *et al.*, 1980; Burgess *et al.*, 1982), but the efficiency of the chromatography is often inferior to separations in the presence of high concentrations of NaCl (Nice *et al.*, 1979; Cooke *et al.*, 1983). The final step in our purification procedure used the Beckman RPSC column (see Step 5) equilibrated with water containing NaCl (0.9%) and adjusted to pH 2.1 with HCl. The proteins were eluted with a shallow gradient (see Fig. 6) of acetonitrile at a flow rate of 0.5 ml/min. Fractions were collected in polypropylene tubes every 1 min, and 10 μ l of Tween 20 solution (2%, v/v, in water) was added to each tube.

Analysis of different molecular forms of purified GM-CSF

Small samples of the biologically active fractions from the reversed-phase h.p.l.c. in the presence of NaCl were analysed by size-exclusion chromatography on a TSK3000 SW column (30 cm $\times$ 7.5 mm internal diam.) equilibrated with ammonium acetate (50 mM; pH 6.5) containing Tween 20 (0.02%). The column was developed at 0.25 ml/min and fractions were collected every 1 min. The column was calibrated with a set of M_r standards, and the apparent M_r of each GM-CSF was estimated by extrapolation (Gough & Gough, 1983). The M_r values of the forms were also determined by SDS/polyacrylamide-gel electrophoresis on 12% polyacrylamide gels (Laemmli, 1970). The protein was detected on the gels by silver staining (Morrisey, 1981), and the biological activity was determined as described previously (Burgess *et al.*, 1977; Metcalf, 1977).

Preparation of ^{125}I -labelled GM-CSF

Purified GM-CSF (from Step 6) was radioiodinated by the method of Tejedor & Ballesta (1982). A sample of GM-CSF containing approx. 1 μ g was concentrated to 60 μ l with a Speedi-vac rotary evaporator. The solution was made up to 0.125 M in KI and buffered with 10 μ l of sodium borate (0.2 M; pH 8.4). Then 1 mCi (10 μ l) of carrier-free $^{125}\text{I}^-$ (Amersham International, Amersham, Bucks., U.K.) was added to the reaction mixture, which was then exposed to chlorine gas for 30 min. The filter pad containing the NaCl and chloramine-T was replaced at 10 min and 20 min. Free iodide was removed by exchanging the ^{125}I -labelled GM-CSF into phosphate-buffered saline (0.14 M-NaCl/0.02 M-sodium phosphate, pH 7.4) on a Sephadex G-25 column (Pharmacia,

PD-10 column). The ^{125}I -labelled GM-CSF was analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), isoelectric focusing in agarose gels (Burgess *et al.*, 1983), microbore reversed-phase h.p.l.c. and the TSK3000 SW column.

Specific radioactivity of ^{125}I -labelled GM-CSF

This was determined by microbore reversed-phase h.p.l.c. as described previously (Nice *et al.*, 1984). A Hewlett-Packard model 1090 high-performance liquid chromatograph fitted with a model 1040 diode-array detector and a 250 μ l autoinjection syringe was used. Separation was obtained on a Brownlee RPP 'guard' column (3 cm $\times$ 2.1 mm internal diam.; 30 nm pore-size silica, C_8 bonded phase) at 45 $^\circ\text{C}$ and a flow rate of 100 μ l/min with a linearly increasing gradient (1%/minute) between 0.9% NaCl (pH 2.1) and acetonitrile. Detection was at 210 nm, with a reference wavelength of 320 nm. A 150 μ l sample of the radioiodinated GM-CSF preparation was injected. Fractions were collected manually at 2 min intervals (allowing for the 50 μ l dead volume between the detector flow cell and the outlet port), and the associated radioactivity was determined with a Packard Autogamma 500C counter with an automatic ^{125}I efficiency-determination programme. The apparent mass of GM-CSF was determined by comparison of the observed peak area with the peak areas of a set of bovine serum albumin standards.

Glycosidase treatment of ^{125}I -labelled GM-CSF

^{125}I -labelled GM-CSF (approx. 1 μCi in 5 ml) was mixed with 5 μ l of sodium acetate buffer [50 mM; pH 5.0; containing Tween 20 (0.02%) and mercaptoethanol (2%, v/v)] and 0.5 μ l of endoglycosidase F (New England Nuclear), endoglycosidase D (Seikagaku, Japan), endoglycosidase H (New England Nuclear) or neuraminidase (Worthington). Each incubation contained 2 munits of enzyme. The incubations were allowed to proceed for 1 h, 2 h or 22 h before the samples were mixed with either isoelectric-focusing sample buffer (O'Farrell, 1975) or SDS/polyacrylamide-gel-electrophoresis sample buffer (O'Farrell, 1975) for analysis.

Protein assays

The total protein estimates for the first four purification procedures were made by the method of Lowry *et al.* (1951), but after that stage there was insufficient protein to obtain an accurate estimate by that procedure. Thus the protein content of fractions eluted from the h.p.l.c. columns was estimated by comparison of the A_{215} with that of a bovine serum albumin standard. Although this method is unlikely to yield the absolute value for GM-CSF protein, it does allow a direct comparison of the biological and specific activity of different preparations.

RESULTS AND DISCUSSION

The discovery of colony-stimulating factor, G-CSF, in endotoxin serum (Burgess & Metcalf, 1980) and subsequently in MLCM (Nicola & Metcalf, 1981) meant that this conditioned medium was a valuable source for at least two distinct haemopoietic regulators, GM-CSF and G-CSF. The initial purification scheme for the isolation of GM-CSF from MLCM (Burgess *et al.*, 1977) was not appropriate for the preparation of G-CSF at the

Table 1. Purification and properties of granulocyte/macrophage colony-stimulating factor from mouse lung conditioned medium

The procedure started with 18 litres of MLCM (Burgess *et al.*, 1977). Steps 2a and 2b were performed with 3-litre batches of MLCM, but the quantities calculated represent an 18-litre batch. Step 3 was performed on the active fractions pooled from six step-2 purifications.

Step	$10^{-6} \times$ Total activity (no. of colonies)	Total protein (mg)	$10^{-8} \times$ Specific activity (colonies/mg)	Purification (-fold)	Yield (%)
1. MLCM	630	31 600	0.0002	1	100
2. (a) Salting-out and (b) Phenyl-Sepharose chromatography	500	15 000	0.0003	1.6	79
3. Concanavalin A-Sepharose (sugar eluate)	125	600	0.002	10	20
4. Ultrogel AcA54	124	63	0.019	95	20
5. Ultrosphere RPSC C ₃ /HFBA	100	0.5	2.0	10 000	16
6. Pharmacia Mono Q	70	0.05	14	70 000	11
7. Ultrosphere RPSC C ₃ /NaCl					
A	12	0.006	20	100 000	2
B	24	0.012	20	100 000	4
C	24	0.012	20	100 000	4
D	12	0.006	20	100 000	2

same time. Indeed, to allow larger volumes of MLCM to be used for the purification of GM-CSF, an $(\text{NH}_4)_2\text{SO}_4$ (50–80%) precipitation step was introduced. However, although this precipitated the GM-CSF, the G-CSF did not precipitate quantitatively. Also, the original procedure for obtaining GM-CSF involved a final preparative gel-electrophoresis procedure, and contamination with gel-related artefacts prevented the efficient radioiodination and analysis of the purified molecule. The development of h.p.l.c. procedures for the purification of low-abundance hydrophobic proteins (Nice *et al.*, 1979; Cooke *et al.*, 1983) such as GM-CSF has led to the development of a new purification procedure which yields several forms of GM-CSF, which are suitable as biological reagents for analysis and receptor studies.

Purification of GM-CSF from mouse lung conditioned medium

The preparation of MLCM with optimal contents of GM-CSF and minimal amounts of mouse serum is described in the original purification procedure (Burgess *et al.*, 1977). The salting-out chromatography using Sepharose CL-6B and $(\text{NH}_4)_2\text{SO}_4$ has been described in detail (Nicola *et al.*, 1983) and is essentially the same as the published procedure. All of the GM-CSF activity free of G-CSF was kept, and the remainder of the GM-CSF activity (which is associated with all of the G-CSF) was recovered from the early-eluted material from the phenyl-Sepharose CL-4B column. The proportion of GM-CSF free of G-CSF was often greater than 80%, but sometimes more than 30% of the total GM-CSF from the salting-out column was still associated with G-CSF. All of this GM-CSF was recovered from the phenyl-Sepharose CL-4B column and pooled with the salting-out fraction. Although these two procedures considerably decreased the amount of protein associated with the GM-CSF, there was some loss of biological activity (Table 1). However, the GM-CSF and G-CSF molecules were completely separated.

A most effective procedure for removing most of the protein from the Step-2 material (Table 1) involved the use of concanavalin A-Sepharose. The isolation of

Fig. 1. Gel filtration of MLCM proteins eluted from concanavalin A-Sepharose on Ultrogel AcA54

The fractions eluted from the concanavalin A-Sepharose column containing GM-CSF were pooled, concentrated over an Amicon YM-10 membrane and chromatographed on the Ultrogel AcA54 column (2.4 cm $\times$ 180 cm). The ability of the fractions to stimulate granulocyte/macrophage colonies from bone-marrow cells (▨) and the protein absorbance at 280 nm (—) are shown. The void volume (V_0) was measured with Blue Dextran and the total volume (V_t) with vitamin B-12.

the glycoprotein fraction allowed the removal of not only 95% of the contaminating proteins, but in particular the albumin, which often makes the final purification of the CSFs particularly difficult. The proportion of GM-CSF binding to concanavalin A-Sepharose varied between preparations (from 40 to 60%), but in most preparations only half of the GM-CSF bound to concanavalin A-Sepharose appeared to be eluted with α -methyl mannopyranoside and α -methyl glucopyranoside. Despite this relatively low recovery, the 5-fold increase in purity of the GM-CSF, coupled with the specific removal of the albumin, justifies its inclusion in the purification scheme.

Fig. 2. Reversed-phase h.p.l.c. of mouse GM-CSF on an Ultrapore RPSC large-pore (C_3 , 30 nm) column, at pH 2.1 in the presence of HFBA

The acetonitrile concentration (----) was increased at 1%/min and the flow rate maintained at 1 ml/min. The protein absorbance was monitored at 280 nm (—) and the GM-CSF content of each fraction was assayed (■) on bone-marrow cells after diluting (1:1000) a sample of each fraction.

The glycoprotein fraction from concanavalin A-Sepharose containing GM-CSF (Table 1) was loaded on to the Ultrogel AcA54 column. Most of the protein was eluted with an apparent M_r greater than 50000 (Fig. 1),

but, in agreement with the previous results with Ultrogel AcA44 (Burgess *et al.*, 1977), the GM-CSF was eluted with an apparent M_r of 29000 (K_{av} = 0.28). The recovery of the GM-CSF was quantitative, and the further 10-fold purification decreased the protein content sufficiently to allow a high-resolution reversed-phase h.p.l.c. procedure.

Although phosphate-buffered saline was used for the gel filtration, it was still possible directly to trace-enrich (Nice *et al.*, 1984) the GM-CSF from the pooled active fractions (80 ml) directly on to the Ultrapore RPSC C_3 . It is possible to perform the Ultrogel AcA54 purification with Tris/HCl (pH 7.0, 20 mM) with equal efficiency. This allows the GM-CSF to be recovered and eluted from calcium phosphate gel (Burgess *et al.*, 1977; Keilin & Hartree, 1938) before h.p.l.c. Although we have not had sufficient experience with this procedure to determine the effect of this extra procedure on overall recovery, it has been useful for the further purification of the GM-CSF which fails to bind to concanavalin A-Sepharose.

Our initial reversed-phase h.p.l.c. was performed on a short-alkyl-chain (C_3) large-pore (30 nm) column packing to improve the recovery of the GM-CSF, even at high protein loads. Several solvents (e.g. propan-1-ol, propan-2-ol and acetonitrile) were tested, but the acetonitrile gradient provided the best protein resolution without compromising GM-CSF recovery. This first reversed-phase h.p.l.c. step was performed with HFBA as an ion-pairing agent. In addition, the poor u.v.-absorption characteristics of this solvent suggested use at a relatively early stage in the h.p.l.c. purification, when there was still sufficient protein present to monitor at 280 nm. The GM-CSF was eluted from the column between 42 and 47 min, slightly behind the major protein peak (Fig. 2).

Fig. 3. Anion-exchange h.p.l.c. of the GM-CSF pooled from the reversed-phase h.p.l.c. in the presence of HFBA (Fig. 2)

The column was equilibrated with Tris/HCl (20 mM; pH 8), and the reversed-phase h.p.l.c. pool was diluted 10:1 with this buffer and loaded directly on to the column. After re-equilibration of the column, the proteins were eluted with a discontinuous NaCl gradient: initially the NaCl concentration (----) was increased to 0.1 M over a 5 min interval, and subsequently to 0.15 M during the next 30 min. The protein was monitored by fluorescence of the tryptophan residues (excitation at 215 nm and emission detected at 340 nm; —). The GM-CSF content of each fraction was measured (■) on diluted (1:1000) samples from each fraction (1 ml) by using the standard bone-marrow assay.

Fig. 4. Electrophoretic analysis of the GM-CSF eluted from the Pharmacia Mono Q column (Fig. 3) on polyacrylamide gel (10%, w/v) in the presence of SDS

The sample (20 μ l) was mixed with an equal volume of SDS sample buffer and 20 μ l of the mixture loaded into each of two lanes for electrophoresis. Half of the gel was silver stained (left panel) and the other lane was sliced into 2 mm segments, each of which was crushed into 1 ml of phosphate-buffered saline containing bovine serum albumin (10 mg/ml), and the proteins were eluted overnight. A mixed-bed ion-exchange resin was added to each eluate (Burgess *et al.*, 1977) for 30 min before sterilization by filtration. These eluates were assayed at several dilutions for GM-CSF by the standard soft-agar bone-marrow-colony assay (Metcalf, 1977), and the results for each fraction are presented graphically in the middle panel of the Figure. The apparent M_r values were estimated by extrapolation from a standard curve obtained by using mobility of the Pharmacia low- M_r markers (right panel: bovine serum albumin, 68 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soya-bean trypsin inhibitor, 20 000).

Most of the proteins were well resolved, and were eluted with high efficiency. Although the GM-CSF was broader than the other protein bands (perhaps reflecting some heterogeneity in the biologically active protein), the recovery was greater than 80%, and more than 95% of the contaminating proteins were removed by this procedure.

Although there was some charge heterogeneity associated with GM-CSF, the earlier purification procedure indicated that ion-exchange chromatography on DEAE-Sephadex was an effective purification procedure (Burgess *et al.*, 1977). Thus the pooled fractions from the reversed-phase h.p.l.c. were loaded directly on to a Pharmacia Mono Q column, after dilution of the HFBA and adjustment of pH, and the proteins were fractionated with a shallow NaCl gradient (Fig. 3). Most of the protein was only weakly retained by this packing and was eluted well before the GM-CSF, which was

eluted underneath a broad 'heterogeneous' protein peak (Fig. 3). Recovery of biological activity from this step was only 68%, but again there was an effective decrease in contaminating protein, and a significant purification of the GM-CSF was achieved. All of the fractions eluted between 0.11 M- and 0.13 M-NaCl (i.e. 15–20 min) were pooled, and the protein heterogeneity and GM-CSF activity were analysed by SDS/polyacrylamide (10%)-gel electrophoresis. Half of the gel was silver stained to detect the proteins; the other half was sliced into 2 mm segments and the GM-CSF extracted for assay. Only three proteins were detected by silver staining (Fig. 4): two of the proteins were associated with GM-CSF activity (at M_r 23 000 and 21 000). Indeed, the specific activity of these two CSFs appeared to be identical, and morphological analysis of the bone-marrow colonies (Metcalf, 1977) indicated that both species yielded the same preparations of pure granulocytic (42%), mixed granulocyte/macrophage (29%) and pure macrophage colonies (27%). There was no biological activity associated with the other protein (M_r 39 000).

Several procedures were used in an attempt to separate the 'two' forms of GM-CSF and to remove the contaminating M_r -39 000 protein. Analysis of the GM-CSF eluted from the Pharmacia Mono Q column by size-exclusion chromatography on a TSK3000 SW column (Fig. 5) yielded a single peak of protein and biological activity at an apparent M_r of 29 000 (in agreement with the results from the Ultrogel AcA54 column; Fig. 1). This was somewhat surprising, as we would have expected to separate the M_r -39 000 protein from the GM-CSF. Thus the usefulness of these h.p.l.c.

Fig. 5. Size-exclusion chromatography of the GM-CSF, eluted from the Pharmacia Mono Q column (Fig. 3), on a Beckman TSK3000 SW column

The protein was monitored by the A_{215} (—) and the GM-CSF (▨) by assay after dilution (1:100) by the soft-agar bone-marrow-colony assay (Metcalf, 1977). The column was calibrated with bovine serum albumin ('68K'), ovalbumin ('43K') and α -lactalbumin ('15K') as M_r markers.

Fig. 6. Final preparative reversed-phase h.p.l.c. of GM-CSF in the presence of NaCl

The pooled fractions from the Pharmacia anion-exchange h.p.l.c. Mono Q column (Fig. 3) were pumped on to the Beckman Ultrasphere RPSC (C_3 , 30 nm), column which had been equilibrated at pH 2.1 with NaCl (0.9%). Elution of proteins from the column was performed with a discontinuous gradient of acetonitrile (—, lower panel). The proteins eluted from the column (—, lower panel) were monitored at 210 nm. The GM-CSF content of each fraction (■, upper panel) was measured by titration after dilution (1:1000) and sterilization, by using the semi-solid bone-marrow-colony assay (Metcalf, 1977). The two major peaks of protein, after 28 and 35 min corresponded to the GM-CSF.

gel-filtration columns for high-resolution analysis is somewhat limited for this particular application, since the electrophoresis results indicated that at least three different proteins were present across the apparently homogeneous protein peak (Fig. 5).

Final purification and separation of multiple forms of GM-CSF was achieved by returning to the Ultrapore RPSC (C_3) column, but replacing the HFBA by NaCl (0.9%, adjusted to pH 2.1 with HCl) and employing a shallow gradient (Fig. 6). Two major peaks of protein (with a leading shoulder on the first peak) were detected. The biological activity was coincident with these two peaks of protein. SDS/polyacrylamide (10%)-gel electrophoresis was used to analyse the proteins in the active fractions (Fig. 7). The two major peaks of biological activity (Fig. 6) contained proteins with of apparent M_r 23000 and 21000 (Fig. 7). Thus four species of GM-CSF (A, B, C, D) were identified and purified by this procedure. The contaminating protein (M_r 39000) was not eluted from the Ultrapore RPSC column in the presence of NaCl, and was only washed from the column when the column was cleaned with HFBA (0.2%). The overall recovery of GM-CSF from the final procedure was essentially quantitative and represented approx. 12% of the initial GM-CSF present in the MLCM. Only 6–12 μ g of each species was produced from the 18 litres of starting MLCM, and this represented a purification of 10^5 -fold for each. The biological properties of all four species were identical, each being capable of stimulating granulocytic, granulocyte/macrophage and pure macrophage colonies in similar proportions to the Stage-6 GM-CSF. [N.B. each of the silver-stained protein bands at M_r 23000 and 21000 detected in the Stage-6 material

(Fig. 4) must consist of two different GM-CSF molecules, which can be separated subsequently on the Ultrapore RPSC (C_3) column in the presence of NaCl (Fig. 6).]

Radioiodination of GM-CSF

The GM-CSF purified previously from MLCM was eluted from a preparative polyacrylamide gel (Burgess *et al.*, 1977) and contained buffer ions, detergent and other impurities from the polyacrylamide, which prevented its detailed analysis and radiolabelling. However, in contrast with the M-CSF molecule (Stanley & Heard, 1977; Guilbert & Stanley, 1980), most methods of radiolabelling significantly decreased the biological activity of mouse GM-CSF (Burgess & Metcalf, 1977). This problem was overcome for G-CSF (Nicola & Metcalf, 1984) by using a gas-phase iodination procedure (Tejedor & Ballasta, 1982), which not only yielded a fully biologically active G-CSF but also attached more than two atoms of ^{125}I to each molecule of G-CSF. By a similar method ^{125}I was incorporated into 1 μ g of GM-CSF sub-species B (Fig. 7) (all GM-CSF sub-species recovered from the Ultrasphere RPSC column could be iodinated to a similar extent). When 1 mCi of ^{125}I was used in the iodination reaction, almost 300 μCi was incorporated into high- M_r material. Further purification of the labelled protein on CM-Sephadex yielded ^{125}I -labelled GM-CSF (essentially free of $^{125}\text{I}^-$ and other low- M_r ^{125}I -labelled contaminants) with a specific radioactivity of 3×10^6 – 5×10^6 d.p.m./pmol. The specific radioactivity was determined quantitatively by using a microbore column (Fig. 8), which we have previously shown to be capable of quantifying less than 5 ng of epidermal growth factor (Nice *et al.*, 1984). Approx. 10% of the ^{125}I -labelled

Fig. 7. SDS/polyacrylamide-(10%)-gel electrophoretic analysis of the purified GM-CSF sub-species, eluted from the reversed-phase h.p.l.c. column in the presence of NaCl (Fig. 6)

A 10 μ l portion of each fraction from the reversed-phase h.p.l.c. column (Fig. 6) was mixed with an equal volume of SDS sample buffer, electrophoresed, fixed and silver-stained. The silver-stained Pharmacia M_r markers are shown in lane (a), and the silver-stained proteins from representative fractions across the GM-CSF protein profile (Fig. 6) are shown in the adjacent lanes (b-e) (the proportion of each sub-species varied between different GM-CSF preparations): (b) GM-CSF A; (c) GM-CSF B; (d) GM-CSF C; (e) GM-CSF D.

GM-CSF was analysed on the reversed-phase h.p.l.c. microbore column, and 71 ng of GM-CSF protein was associated with 1.7×10^7 d.p.m. of 125 I. No loss of biological activity was detected as a result of the labelling procedure. This 125 I-labelled GM-CSF was suitable for GM-CSF receptor analysis (Walker & Burgess, 1985).

Glycosidase treatment of 125 I-labelled GM-CSF

The 125 I-labelled GM-CSF was initially analysed by isoelectrofocusing on an agarose gel, and the results were compared with the distribution of biological activity of partially purified (Stage 2; Burgess *et al.*, 1977) MLM GM-CSF (Fig. 9). The biological activity of partially purified GM-CSF focused between pH 5.2 and pH 4.2 (Fig. 9, broken line). The untreated 125 I-labelled GM-CSF also focused between pH 4.4 and pH 5.4, indicating that the purification procedure and subsequent radioiodination had not significantly altered the charge distribution of the GM-CSF. Both the GM-CSF biological activity and 125 I-labelled GM-CSF focused as distinct charge species, corresponding to discrete charge differences [e.g. sialic acid heterogeneity (Ayasawa *et al.*, 1979; Nicola *et al.*, 1979; Bazill *et al.*, 1983)]. On

treatment of the 125 I-labelled GM-CSF and a partially purified GM-CSF preparation with neuraminidase, both the 125 I-GM-CSF and biological-activity profiles shifted to a higher pH. The neuraminidase-treated 125 I-GM-CSF focused sharply (between pH 5.2 and pH 5.4; Fig. 9) and corresponds to the biological activity of the neuraminidase-treated GM-CSF, which focussed between pH 5.2 and pH 5.5 (continuous line, Fig. 9).

The 125 I-GM-CSF was analysed further by SDS/polyacrylamide-gel electrophoresis (Fig. 10). The 125 I-labelled GM-CSF-B was treated separately with neuraminidase, endoglycosidase H, endoglycosidase F and endoglycosidase D. Isoelectric focusing and SDS/polyacrylamide-gel-electrophoretic analysis indicated that endoglycosidase H or endoglycosidase D failed to

Fig. 8. Determination of the specific radioactivity of 125 I-labelled GM-CSF by microbore reversed-phase h.p.l.c.

A 100 μ l sample of the 125 I-labelled GM-CSF or 100 μ l of phosphate-buffered saline was loaded on to a Brownlee RPP guard column (2.1 mm internal diam. $\times$ 3 cm) equilibrated at 45 $^{\circ}$ C with water/HCl adjusted to pH 2.1 and containing NaCl (0.9%). The protein was monitored at 210 nm upper panel: —, GM-CSF, ---, blank) and the 125 I by scintillation counting (lower panel). The protein was eluted from the column with a linearly increasing gradient of acetonitrile (1%/min) at a flow rate of 100 μ l/min. Fractions were collected at 2 min intervals. The apparent mass of 125 I-labelled GM-CSF was determined by comparison with the peak area for the A_{210} of standard amounts of bovine serum albumin.

remove carbohydrate from the ^{125}I -labelled GM-CSF, but neuraminidase and endoglycosidase F both removed carbohydrate side chains. The neuraminidase decreased the M_r of ^{125}I -labelled GM-CSF to 19000, and the endoglycosidase F to 16500 (Fig. 10). Treating the ^{125}I -labelled GM-CSF with both enzymes did not lower the M_r of the GM-CSF any further. Treatment of the M_r -23000 ^{125}I -labelled GM-CSF C sub-species (Figs 6 and 7) yielded identical results (i.e. this sub-species yielded one of M_r 19000 after neuraminidase treatment and 16500 after treatment with endoglycosidase F). Interestingly, the lowest M_r generated for the ^{125}I -labelled GM-CSF molecule after glycosidase treatment is identical with the lowest- M_r subunit reported for glycosidase-treated M-CSF (also called CSF-1) (Das & Stanley, 1982).

The single M_r species (16500) after endoglycosidase treatment suggests that all of the sub-species share a polypeptide chain of the same length; however, the reason for the different elution positions on reversed-phase h.p.l.c. may be modification other than glycosylation. This is in agreement with the analysis of the gene for mouse GM-CSF, which indicates that there is only a single copy (Gough *et al.*, 1984). The apparent M_r of the glycosidase-treated GM-CSF is slightly larger than the size predicted from the cDNA sequence reported by Gough *et al.* (1984). This difference may be due to residual carbohydrate, anomalous electrophoretic behaviour or an uncleaved precursor sequence at the *N*-terminus. Although the *N*-terminal sequence of asialo-(mouse GM-CSF) has been determined (Sparrow *et al.*, 1984), attempts to sequence the *N*-terminus of the untreated GM-CSF purified in this study yielded only the first four amino acids, Ala-Pro-Thr-Arg (Table 2). These four residues correspond to a precursor segment

Fig. 9. Isoelectric-focusing analysis of both untreated and neuraminidase-treated ^{125}I -labelled GM-CSF and partially purified GM-CSF

Partially purified GM-CSF was subjected to isoelectric focusing (with Pharmalytes, pH 4.5–6) on a polyacrylamide gel, before (---) and after (O—O) treatment with neuraminidase. After focusing, the pH of the gel was recorded with a surface electrode and the gel was sliced into equal segments. The GM-CSF was eluted from the crushed gel and the activity determined by the standard bone-marrow-colony assay.

Fig. 10. Electrophoretic analysis of glycosidase-treated ^{125}I -labelled GM-CSF

The ^{125}I -labelled GM-CSF was untreated (lane a), treated with neuraminidase (lane b) or treated with endoglycosidase F (lane c) and analysed by SDS/polyacrylamide (10%)-gel electrophoresis. A sample of the ^{125}I -labelled GM-CSF was incubated for 22 h at pH 5 with no enzyme, or 2 munits of the appropriate enzyme. The apparent M_r values of the ^{125}I -labelled proteins were: lane a, 21000; lane b, 19000; lane c, 16500.

Table 2. Automated Edman degradation of GM-CSF from MLCM

Automated amino acid sequence analysis was performed with an Applied Biosystems sequencer (model 470A), and amino acid phenylthiohydantoin was analysed by h.p.l.c. by the procedure described elsewhere (Simpson *et al.*, 1985). Samples (67%) from each cycle were analysed by h.p.l.c. and the yields of amino acid phenylthiohydantoin were normalized to 100% injection.

Cycle no.	Amino acid phenylthiohydantoin	Yield (pmol)
1	Alanine	7
2	Proline	2
3	Threonine	2
4	Arginine	2

predicted by the cDNA clone for mouse GM-CSF from a T-lymphocyte cell line (Gough *et al.*, 1984). This segment starts six amino acids before the *N*-terminus determined for the asialo-GM-CSF (Sparrow *et al.*, 1985), suggesting that the neuraminidase treatment may have modified the *N*-terminus of a small proportion of the asialo-GM-CSF, allowing an internal fragment to be sequenced.

Analysis of the ^{125}I -labelled GM-CSF indicated that we

were able to attach 2–3 atoms of ^{125}I to the GM-CSF molecule without affecting its biological activity. Although this is considerably less than the 4–6 atoms incorporated into M-CSF (CS-1; Guilbert & Stanley, 1980), this derivative has been suitable for the detection and analysis of mouse GM-CSF receptors (Walker & Burgess, 1985).

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