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Purification and partial amino acid sequence of asialo murine granulocyte–macrophage colony stimulating factor

(neuraminidase treatment/HPLC/sequence microanalysis/mouse lung-conditioned medium)

LINDSAY G. SPARROW*, DONALD METCALF†, MICHAEL W. HUNKAPILLER‡, LEROY E. HOOD‡, AND ANTONY W. BURGESS§

*Division of Protein Chemistry, Commonwealth Scientific and Industrial Research Organisation, Parkville 3052, Victoria, Australia; †Walter and Eliza Hall Institute for Medical Research and §Tumor Biology Branch, Ludwig Institute for Cancer Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia; and ‡Division of Biology, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **A procedure utilizing reversed-phase high-performance liquid chromatography is described for the purification of asialo granulocyte–macrophage colony stimulating factor (asialo-GM-CSF) from mouse lung-conditioned medium. In the purification, the partially purified factor was treated with neuraminidase to reduce charge heterogeneity due to variable degrees of sialation. Three active forms of the asialo factor were separated by the final reversed-phase liquid chromatography step. These each gave a single major band and several minor bands on polyacrylamide gel electrophoresis and had similar amino acid compositions. The specific activity of purified murine asialo-GM-CSF was approximately 8 × 10^9 colonies per mg of protein. Amino acid sequence determination of the major form gave a single amino-terminal sequence, which has been used to develop oligonucleotide probes for the isolation of two cDNA clones encoding GM-CSF. The nucleotide sequence of these two clones gave a deduced amino acid sequence almost identical with that determined for the amino terminus of asialo-GM-CSF and an amino acid composition very similar to that for asialo-GM-CSF.**

Granulocyte–macrophage colony stimulating factor (GM-CSF) is a glycoprotein that is required for the *in vitro* proliferation and differentiation of precursor cells to give colonies of granulocytes and macrophages (1–4). Comparable hemopoietic regulatory factors are macrophage CSF (M-CSF; ref. 5), granulocyte CSF (G-CSF; ref. 6), multi-CSF (ref. 7; also called interleukin-3 or IL-3, see ref. 8) and erythropoietin (9), which regulates the formation of erythrocytes. These proteins have proved difficult to purify because of their low abundance and carbohydrate heterogeneity; this has generally prevented more than a few micrograms of any of these growth factors from being purified. Recently, preliminary amino acid sequence information was obtained for multi-CSF (IL-3) (10), and the subsequent molecular cloning of the cDNA coding for this protein (11) has yielded the full amino acid sequence.

The identification and structural analysis of both mouse and human GM-CSF genes and their related products are essential steps in the understanding of the mechanism of action of the hemopoietic regulators and their role in the etiology of leukemia (12). The amino acid sequence data for murine GM-CSF, reported here, has aided in the identification of cDNA clones for this molecule (13) and should eventually lead to the isolation of the corresponding genes encoding GM-CSF.

A purification of GM-CSF from mouse lung-conditioned medium (MLCM) has been reported (14). This used a number of conventional chromatographic steps, followed by preparative PAGE, and led to apparently homogeneous material as judged by PAGE in both denaturing and nondenaturing conditions. However, the small amount of material available prevented the effective structural analysis of this molecule.

GM-CSF has a broad isoelectric range from 4.1 to 5.2 due largely to heterogeneity in glycosylation, specifically sialation; treatment with neuraminidase greatly reduces the charge heterogeneity and raises the pI from 5.2 to 5.5 (15). Incorporation of neuraminidase treatment in the purification protocol thus significantly improves the efficiency of charge-dependent separation steps.

This report describes the purification of asialo-GM-CSF from MLCM by an improved protocol incorporating a neuraminidase treatment and using reversed-phase HPLC (RP-HPLC) as a final step instead of preparative gel electrophoresis. The purified factor is essentially homogeneous by PAGE and yielded a unique amino-terminal amino acid sequence for murine GM-CSF.

MATERIALS AND METHODS

Unless otherwise specified, all buffers contained Triton X-100 (0.01%, wt/vol), and all operations were carried out at 4°C. MLCM was prepared as described (14) by using the lungs from C57BL mice previously treated with endotoxin (16).

Purification of Asialo-GM-CSF. MLCM (10.5 liters) was subjected, in three approximately equal batches, to heat and then dialyzed against water and fractionated on calcium phosphate gel as described (15). Two of the three batches also were carried through ion-exchange chromatography on DEAE-cellulose as described (14). The three batches then were combined, concentrated by ultrafiltration in a Diaflo cell using a YM10 membrane (Amicon), and dialyzed against 0.2 M sodium acetate, 1 mM in Ca^{2+}, Mg^{2+}, and Mn^{2+}, pH 5.0, to give partially processed conditioned medium.

Concanavalin A-Sepharose Chromatography. The partially processed conditioned medium was then chromatographed on a column of concanavalin A-Sepharose (1.5 × 27 cm; Pharmacia) as described (14).

Neuraminidase Treatment. The glycoproteins that bound to concanavalin A-Sepharose were eluted with a solution of 0.05 M α-methyl mannoside in the above buffer, concentrated by ultrafiltration, and dialyzed in the Diaflo cell against 0.05 M sodium acetate (pH 5.0). The proteins then were incubated overnight at 37°C with neuraminidase from *Clostridium perfringens* (type X; Sigma) at a protein-to-enzyme ratio of 10 mg/unit of enzyme activity. At this stage material from a separate preparation, starting with 3 liters of MLCM and

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Abbreviations: CSF, colony stimulating factor; GM-CSF, granulocyte (G)-macrophage (M) CSF; MLCM, mouse lung-conditioned medium; RP-HPLC, reversed-phase HPLC; > PhNCS, phenylthiohydantoin.

taken through the same purification steps, was combined with the above material.

DEAE-Cellulose Chromatography. The neuraminidase-treated material was dialyzed against 0.01 M Tris chloride (pH 7.4) and chromatographed on a column of DEAE-cellulose (1.5 × 80 cm, Whatman DE-52) equilibrated with the same buffer. After sample application, the column was eluted with 200 ml of the same buffer, followed by a linear gradient of 0–0.17 M sodium chloride in a total volume of 1000 ml of the same buffer at a flow rate of 8 ml/hr. Finally, the column was eluted with 0.5 M sodium chloride in the same buffer.

Gel Filtration. Active fractions were combined, concentrated to a small volume, and loaded on a column of Ultrogel AcA44 (LKB) (2.3 × 140 cm) equilibrated with 0.01 M Tris chloride/0.05 M sodium chloride, pH 7.4, and eluted with the same buffer at a flow rate of 8.5 ml/hr. Active fractions were then concentrated in a Diaflo cell (YM10 membrane) for fractionation by RP-HPLC.

Reversed-Phase HPLC. A Waters Associates HPLC system consisting of two M6000A pumps, an M660 solvent programmer, a U6K injector, an M450 detector and a μ-Bondapak C_{18} column (3.9 × 300 mm) was used for the chromatography. Separations were performed at ambient temperature at a flow rate of 1 ml/min. Fractions were collected manually in siliconized glass tubes, and aliquots were removed and diluted into fetal calf serum (5%, vol/vol) in sodium chloride (0.9%) solution for assay.

PAGE. $NaDodSO_4$/PAGE was carried out by the method of Laemmli (17). Gel slabs were 0.7 mm thick and contained 15% (wt/vol) acrylamide. Samples were heated for 2 min at 100°C in sample buffer (no reductant) before loading. Silver staining was carried out by the method of Merril *et al.* (18).

Biological Assay for GM-CSF Activity. This was carried out as described (14, 19) by determining G–M colony formation in cultures of 75,000 C57BL bone marrow cells.

Protein Estimation. Protein was estimated during the purification by the method of Lowry *et al.* (20), with bovine serum albumin as a standard. The protein yield for purified GM-CSF was determined by amino acid analysis.

Amino Acid Analysis. Aliquots of protein (≈20 pm) were hydrolyzed for 24 hr at 110°C in 6 M HCl containing 0.1% phenol and analyzed with a fluorescence-based amino acid analyzer using *o*-phthalaldehyde (21). No corrections were made for losses of serine or threonine during hydrolysis.

Amino-Terminal Sequence Analysis. Amino-terminal sequence determination was carried out on a gas-phase sequencer as described by Hewick *et al.* (22). Protein in 0.1% CF_3COOH/H_2O/acetonitrile from the RP-HPLC was dried down in a siliconized glass tube for transport from Melbourne to Los Angeles. The tube was then extracted with, in order, 60 μl of 1% $NaDodSO_4$, 30 μl of distilled water, and 60 μl of 25% CF_3COOH. All extractions were loaded onto the precycled glass filter, air dried, and loaded into the sequencer for analysis. Identification of phenylthiohydantoin (> PhNCS)-amino acid derivatives was carried out by HPLC (23).

RESULTS

Neuraminidase Treatment of GM-CSF. Treatment of MLCM with neuraminidase from *Cl. perfringens* yielded a CSF with a higher and narrower isoelectric range (unpublished data) with less polydispersity (unpublished data) than that of untreated MLCM. The isoelectric range for untreated GM-CSF was ≈4.1–5.2, whereas the bulk of the neuraminidase-treated material focused between pH 5.2 and 5.5. Comparison of the morphology of cells (24) in colonies stimulated by GM-CSF or asialo-GM-CSF showed no significant difference, indicating that the *in vitro* biological activity of the two molecular species was identical.

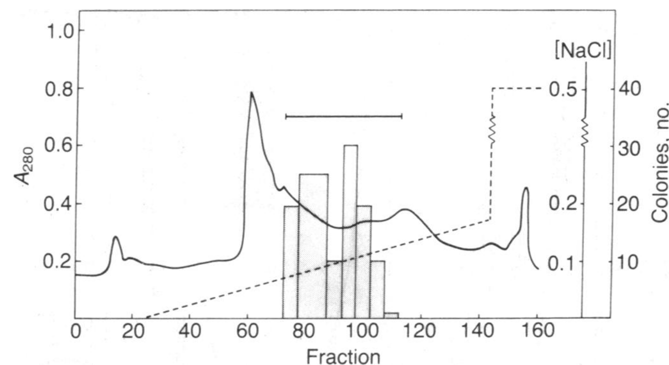

FIG. 1. Elution profile for chromatography on DEAE-cellulose. Aliquots (20 μl) were removed from every fifth fraction (8 ml) for biological assay (shaded columns). Absorbance at 280 nm (——) was measured with distilled water as reference. Fractions were pooled as indicated (|——|). ------, Applied salt gradient.

Purification of Asialo-GM-CSF. The purification of asialo-GM-CSF was based on the previously described method (14) in which the major fractionation steps were chromatography on DEAE-cellulose, concanavalin A-Sepharose, and Ultrogel AcA44, followed by preparative PAGE. In order to reduce contaminating protein as far as possible before neuraminidase treatment, fractionation on concanavalin A-Sepharose was carried out first, followed by neuraminidase treatment and then chromatography on DEAE-cellulose and Ultrogel AcA44; preparative PAGE was replaced by RP-HPLC. The elution profile for DEAE-cellulose chromatography of neuraminidase-treated material is shown in Fig. 1, while that for the gel filtration of the resulting active fractions, indicating an apparent M_r of 33,000 [cf., a value of 29,000 in a previous study (14)], is shown in Fig. 2. The elution profile for the final RP-HPLC step is shown in Fig. 3. The complete purification of asialo-GM-CSF starting from step 2 MLCM is summarized in Table 1. Material from two separate preparations was combined after the neuraminidase treatments; however, the total activity at this stage was significantly higher than expected (shown in parentheses in Table 1) from the activities of the separate preparations. As neuraminidase treatment does not change the *in vitro* activity (15), this discrepancy may be due to variability in the bioassays, which also would have been responsible for the

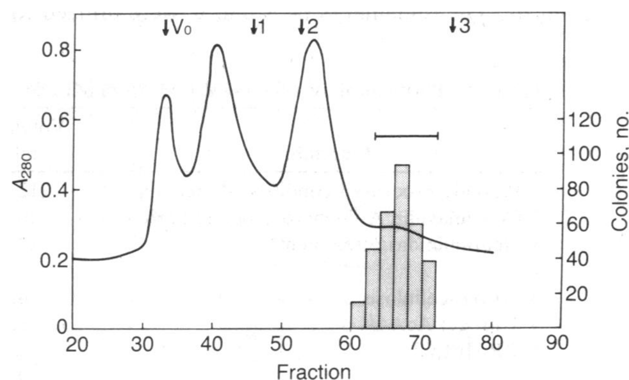

FIG. 2. Elution profile for gel filtration on Ultrogel AcA44. Aliquots (10-μl) were removed from every second fraction (4 ml) for biological assay (shaded columns). Absorbance at 280 nm (——) was measured with distilled water as reference. Fractions were pooled as indicated (|——|). Molecular weight markers were: V_0, blue dextran; 1, bovine serum albumin (67,000); 2, ovalbumin (43,000); 3, chymotrypsinogen A (26,000).

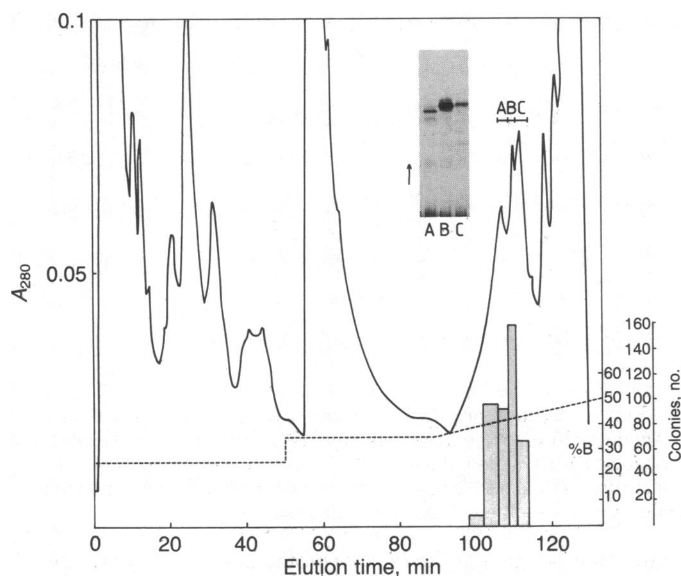

FIG. 3. Purification of asialo-GM-CSF by RP-HPLC of pooled active fractions from Ultrogel AcA44 chromatography. Buffers: A, 20% acetonitrile/0.05% CF_3COOH; B, 80% acetonitrile/0.05% CF_3COOH. Concentrated, active fractions (500 μl) were injected onto the column, which was equilibrated at 25% buffer B. ------, Applied solvent program. Aliquots (20 μl) were removed from each fraction for biological assay (shaded columns). (*Inset*) $NaDodSO_4$/PAGE of active fractions A, B, and C from the RP-HPLC step; 5–10% of each fraction was dried down before dissolving in sample buffer as described.

slight increase in activity between steps 4 and 5.

Asialo-GM-CSF separated as a complex peak on RP-HPLC. When the most active fractions (A, B, and C) were subjected to $NaDodSO_4$/PAGE (Fig. 3 *Inset*), all showed a single major band and several minor bands of silver-stained protein. The position of the major bands was comparable with that demonstrated for the biological activity in a previous study (14). However, the major bands (A, B, and C) from the three RP-HPLC fractions (Fig. 3) differed slightly in their mobility on $NaDodSO_4$ gels, and that for fraction B was significantly heavier than those for A and C, although approximately equal protein loads were used for each track. Fraction B had the highest specific activity and was obtained in a final yield of 14%. The total yield for A, B, and C was 27%.

Aliquots of each of A, B, and C were subjected to amino acid analysis. The remainders of A and C were utilized for further chromatographic experiments, while that for B was subjected to amino-terminal sequence analysis.

Amino Acid Composition of Asialo-GM-CSF. The amino acid composition of asialo-GM-CSF was determined after HCl hydrolysis of suitable aliquots of fractions A, B, and C. The analyses were carried out on a fluorescence-based amino acid analyzer and, because of the inherent limitations of this system, failed to provide values for proline and tryptophan. Values for methionine and cysteine also were not obtained because of the difficulties of amino acid analysis at high sensitivity. The limited amino acid composition of asialo-GM-CSF (fractions A, B, and C) is shown in Table 2.

Amino-Terminal Sequence Analysis of Asialo-GM-CSF. The remainder of fraction B, amounting to ≈300 pmol of protein by amino acid analysis, was subjected to amino acid sequence analysis with a gas-phase sequencer. A single amino acid sequence could be clearly read for 34 residues with 31 residues being readily assigned (Fig. 4, rows a). The amino-terminal residue itself could not be identified, as > PhNCS derivatives of isoleucine, valine, and serine were all evident after the first cycle. The yields of > PhNCS–amino acid derivatives identified for each position throughout this run are shown in Fig. 5, which also shows that the initial yield was only 20 pmol while the repetitive yield was 95.5%. It should be noted that, due to adsorptive losses, only 50 pmol of the original 300-mol sample was recovered from the siliconized glass tube.

DISCUSSION

Structural studies on GM-CSF have been hampered by lack of material because of the extremely small amounts of active protein in each of the richest sources. The purification described above yielded a preparation (fraction B) with a specific activity of 7.9×10^9 colonies per mg that gave essentially a single band on $NaDodSO_4$/PAGE and yielded a single amino acid sequence. The material from the earlier purification protocol (14), which also gave a single band on $NaDodSO_4$/PAGE, had a specific activity of $\approx 7 \times 10^7$ colonies per mg. Comparison of the two different purification protocols shows the major difference to be the increased efficiency of RP-HPLC (×40 purification) compared to preparative PAGE (×2 purification) as the final step. The different order of steps in the two protocols makes it difficult to directly assess the effect of neuraminidase treatment on the efficiency of the ion-exchange fractionation.

Using oligonucleotide probes derived from the partial amino acid sequence of asialo-GM-CSF (Fig. 4, rows a), Gough *et al.* (13) have isolated two cDNA clones from a mouse lung

Table 1. Purification of asialo-GM-CSF from MLCM

Fraction	Volume, ml	Total protein, mg	Total activity, Col × 10^{-6}	Specific activity, Col × 10^{-6} per mg
1 Partially processed conditioned medium	212	763	848	1.1
2 Concanavalin A-Sepharose-bound protein	10	190	565	3.0
3 Neuraminidase treatment*	22.5	315	1499	4.8
			(936)†	(3.0)†
4 DEAE-cellulose	6.2	56	426	7.6
5 Ultrogel AcA44	1.6	3.0	529	176
6 RP-HPLC‡				
A	2.7	0.011§	33.8	3000
B	2.0	0.007§	58.8	7900
C	3.0	0.011§	24.2	2100

Col, GM colony formation.
*Material from two separate preparations combined at this step.
†Calculated from the activities of the separate preparations at step 2.
‡From 0.5 ml of step 5 material.
§By amino acid analysis.

Table 2. Amino acid composition of GM-CSF

Amino acid	Fractions from RP-HPLC			From cDNA
	A	B	C	
Lys	8.0	9.9	8.7	11
His	1.8	2.3	2.3	1
Arg	5.1	4.9	5.3	3
Trp	ND	ND	ND	1
Cys	ND	ND	ND	4
Asp	11.3	9.1	10.5	11
Thr	9.6	12.2	10.0	14
Ser	9.6	6.5	7.7	5(4)*
Glu	14.0	15.9	15.1	15
Pro	ND	ND	ND	7
Gly	8.1	6.8	8.9	3(4)*
Ala	7.6	7.0	6.3	5
Val	6.5	6.3	7.3	8
Met	ND	ND	ND	2
Ile	4.5	4.3	3.3	6
Leu	7.3	9.3	9.7	11
Tyr	3.7	3.4	3.4	4
Phe	6.8	6.1	5.4	7

Amino acid compositions are calculated as residues per mole (118 residues) assuming values for cysteine, methionine, proline, and tryptophane from the cDNA sequence (13). ND, not determined. *Uncertainties in these values are due to ambiguities in the cDNA sequence.

cDNA library and have demonstrated that these encode murine GM-CSF. From the nucleotide sequence of these clones, the complete amino acid sequence of GM-CSF has been deduced; the first 34 residues of this deduced sequence are shown in Fig. 4, rows b. This sequence is essentially identical for the first 34 residues with that obtained by direct protein sequencing. At three positions, 1, 24, and 32, no > PhNCS–amino acid derivative could be assigned. The cDNA-derived amino acid sequence shows isoleucine at position 1, which is consistent with the > PhNCS derivative of isoleucine being among the > PhNCS–amino acid derivatives identified after the first sequencing cycle. At position 32, the > PhNCS derivative of glutamic acid was identified but was ascribed to overlap from glutamic acid at positions 29 and 30.

The two sequences differ at two other positions, 2 and 4, underlined in Fig. 4. After cycle 2, the > PhNCS derivatives of threonine and isoleucine were both present, but because the derivative of isoleucine was also identified after cycle 1, its presence at cycle 2 was ascribed to carry over and ignored. After cycle 4, the > PhNCS derivative of glutamic acid was obtained but no > PhNCS derivative of threonine, as expected from the cDNA sequence, and there seems to be

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                                                10
a   ?   Thr Val Glu Arg Pro Trp Lys His Val Glu Ala Ile
b   Ile Ile Val Thr Arg Pro Trp Lys His Val Glu Ala Ile

                                    20
a   Lys Glu Ala Leu Asn Leu Leu Asp Asp Met  ?  Val Thr Leu
b   Lys Glu Ala Leu Asn Leu Leu Asp Asp Met Pro Val Thr Leu

        30
a   Asn Glu Glu Val  ?  Val Val
b   Asn Glu Glu Val Glu Val Val
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FIG. 4. Amino-terminal sequence of GM-CSF. Rows: a, determined on gas-phase sequencer; b, deduced from the sequence of cDNA clones pGM37 and pGM38 (13). Differences between the two sequences are underlined; ? indicates no assignment made.

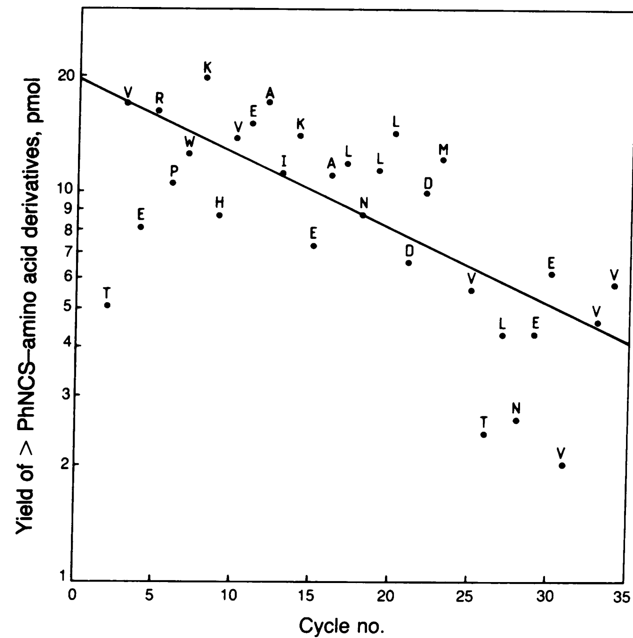

FIG. 5. Yields of > PhNCS–amino acid derivatives identified during sequences of asialo-GM-CSF.

a genuine difference between the two sequences at position 4. Threonine 4 may be modified post-translationally to give an amino acid residue mistakenly identified as glutamic acid during protein sequencing, or perhaps variants of this molecule exist in the mouse population. Such variants could arise through multiple alleles or through polymorphism at a single allele. However, Gough *et al.* (13) have shown that multiple alleles of the GM-CSF gene do not exist in the murine genome.

The amino acid composition of GM-CSF, deduced from the nucleotide sequence, is shown in Table 2. There is qualitative agreement between this composition and that determined for asialo-GM-CSF by direct amino acid analysis of fractions A, B, and C from RP-HPLC. Discrepancies in the values for arginine and glycine can be explained by baseline variations in the arginine region of the amino acid chromatogram and the presence of glycine as a common contaminant in our laboratory at the time. The amino acid compositions of fractions A and C are qualitatively the same as that for fraction B, given the limitations of the technique. Fractions A and C also showed substantial biological activity, and gave each a single band on $NaDodSO_4$/PAGE (Fig. 3); the major bands for fractions A, B, and C are all slightly different in mobility on $NaDodSO_4$/PAGE. However, the specific activities of fractions A and C are significantly lower than that of fraction B. The similarity in amino acid compositions of the three fractions suggests that they are three forms of asialo-GM-CSF, differing in relative activities. Multiple forms of native GM-CSF, separable by RP-HPLC or $NaDodSO_4$/PAGE, have been observed (unpublished data). The differences between these forms appear to reside in differences in the patterns of glycosylation, which also may be responsible for the differential silver staining of the three forms.

Comparison of the amino acid composition and the partial amino acid sequence of fraction B with the composition and sequence of GM-CSF derived from the two cDNA clones (13) confirms that the protein isolated and sequenced in this study is, in fact, asialo-GM-CSF. Such confirmation is particularly important in view of the low yield at cycle 1 of the sequencer run, although low initial yields during sequencer runs are well documented (25). The agreement in the amino acid compositions shows that GM-CSF is the predominant

protein of fraction B, rather than a minor component in a mixture with an amino-terminally blocked protein not detected in amino acid sequencing.

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