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LYMPHOKINE PURIFICATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography has been used to purify to homogeneity two different lymphokines. Human IL-2 was purified on a C_8 reversed-phase column in pyridine–acetate–propanol followed by chromatography on a C_{18} reversed-phase column in trifluoroacetic acid–acetonitrile. Protein sequence analysis of *in situ*-generated cyanogen bromide peptides obtained from this preparation established the homogeneity of this material and confirmed the amino acid sequence predicted from the published DNA sequence. Murine CSF-2α was purified on a C_{18} reversed-phase column in trifluoroacetic acid–acetonitrile followed by chromatography on the same column in pyridine–acetate–propanol. The final preparation yielded a single band on a sodium dodecyl sulfate–polyacrylamide gel with a molecular weight of 24,500.

INTRODUCTION

Lymphokines, including interleukin-2 (IL-2) and colony-stimulating factors (CSF), control to a large extent the activities of the cells involved in generating an immune response (B cells, T cells and macrophages). The purification of these compounds has been of interest in order to obtain material of sufficient purity for protein sequencing and to study more unambiguously the effects of these factors in complex biological systems, especially since the source of most lymphokines is cultured cell supernatants that contain several distinct lymphokine activities.

Reversed-phase high-performance liquid chromatography (HPLC) has proven to be an indispensible tool to use for the realization of this goal. This method was first shown to be useful for the purification of leukocyte interferon[1,2]. More recently, the purification of interleukin-3 (IL-3)[3], hematopoietic cell-growth factor (HCGF)[4], Gibbon ape IL-2[5] and human IL-2[6], has involved at least one step of reversed-phase HPLC. We report the reversed-phase HPLC procedures that we have used to purify to homogeneity two different lymphokines, human IL-2, and one of the murine colony-stimulating factors, CSF-2α. In addition, chemical characterization of these lymphokines is reported.

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EXPERIMENTAL

Lymphokine production and assays for biological activity

Human IL-2 production by the T-cell leukemia cell line Jurkat-H33-JA1 was stimulated by the lectin phytohemagglutinin (PHA 1%) in the presence of phorbol myristate acetate (10 ng/ml, Sigma, St. Louis, MO, U.S.A.). Measurement of IL-2 activity was based on the requirement of cultured T-cell lines for IL-2[8]. One unit of activity corresponded to the amount of factor required to generate 50% of maximal [^{3}H]thymidine incorporation into CTLL cells, an IL-2 dependent murine T-cell line.

We have recently resolved the colony stimulating activity elaborated by the murine T-cell lymphoma line, LBRM-33, into three distinct activities by ion-exchange chromatography (CSF-2α, CSF-2β and CSF-2γ)[9]. CSF-2α production by LBRM-33 cells was stimulated by 1% PHA[10]. Measurement of CSF-2α activity was based on the requirement of a bone-marrow derived murine cell line for this factor[11] (FDC-P2 cells, kindly provided by T. M. Dexter). One unit of activity corresponded to the amount of factor required to generate 50% of maximal [^{3}H]thymidine incorporation into FDC-P2 cells.

Preliminary purification

IL-2 was precipitated from the Jurkat cell conditioned medium by the stepwise addition of ammonium sulfate to 30% and then to a final 80% of saturation. The 80% precipitate was dialyzed against 0.05 *M* sodium chloride, 5 m*M* sodium citrate pH 5.5 and applied to a carboxymethyl (CM) Sepharose column (Pharmacia, Uppsala, Sweden). Column dimensions were 20 × 2.5 cm I.D. A linear gradient from 0 to 0.5 *M* sodium chloride was initiated at a flow-rate of 60 ml/h. IL-2 was eluted at 0.25 *M* sodium chloride, as described[6]. Active fractions from the CM Sepharose column were dialyzed against 20 m*M* sodium chloride, 2.5 m*M* Hepes, pH 7.4, and applied to a 6 × 1.5 cm I.D. diethylaminoethyl (DEAE) Sephacel column (Pharmacia) at 60 ml/h. IL-2 was eluted in the flow-through fraction from the column.

CSF-2α was precipitated from LBRM-33 cell-conditioned medium following sequential addition of ammonium sulfate to 30% then 50% and finally to 80% of saturation. The 80% precipitate was dialyzed against 10 m*M* Tris pH 8.0 and applied to DEAE Sephacel. Column dimensions were 10 × 2.5 cm I.D. The column was operated at a flow-rate of 45 ml/h. CSF-2α was not bound by the column and was found in the flow-through fraction. Active material from the DEAE column was dialyzed against 10 m*M* sodium citrate 100 m*M* sodium chloride (pH 3.5) and applied to a 10 × 2.5 cm I.D. sulfylpropyl Sephadex C-50 (SP) column (Pharmacia). A linear gradient from 0.1 to 0.6 *M* NaCl was initiated at 45 ml/h. CSF-2α was eluted from this column by 300 m*M* sodium chloride, as described[9].

Reversed-phase HPLC

HPLC was performed with a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph, equipped with two model M-45 pumps, a model 720 system controller and a model 441 absorbance detector monitoring at 214 nm. Large sample volumes were pumped onto columns with a Milton Roy minipump (Lab. Data Control, Riviera Beach, FL, U.S.A.). Solvents were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.).

The IL-2 containing material from the DEAE Sephacel column was made 0.9 *M* in acetic acid and pumped directly onto a 25 cm × 9.4 mm I.D. Whatman Magnum 9 Protesil 300 C_8 reversed-phase column (Whatman, Clifton, NJ, U.S.A.) that had been equilibrated in solvent A (0.9 *M* acetic acid, 0.2 *M* pyridine, pH 4.0). Solvent B was 60% *n*-propanol in 0.9 *M* acetic acid, 0.2 *M* pyridine. The gradient of solvent B went from 0 to 20% B in 10 min and from 20% to 84% B in 110 min at a flow-rate of 0.7 ml/min. IL-2-containing fractions from the C_8 column were diluted 1:3 (v/v) with 0.1% (v/v) trifluoroacetic acid (TFA) in water and applied to a 30 cm × 3.9 mm I.D. Waters C_{18} μBondapak column previously equilibrated in 0.1% TFA and eluted with a 1%/min gradient of acetonitrile (0.1% v/v TFA) at a flow-rate of 1 ml/min.

The fractions containing CSF-2α from SP-Sephadex chromatography were made 0.1% in TFA and pumped directly onto a 30 cm × 3.9 mm I.D. Waters C_{18} μBondapak reversed-phase column previously equilibrated in 0.1% TFA in water. Proteins were eluted by a linear gradient of acetonitrile (1%/min, as described above). The peak fraction from the TFA/acetonitrile chromatogram was diluted 1:3 with 0.9 *M* acetic acid, 0.2 *M* pyridine and applied to the same C_{18} column previously equilibrated with 0.9 *M* acetic acid, 0.2 *M* pyridine. The same gradient of *n*-propanol as described for Il-2 purification was then established.

Analysis

Aliquots from HPLC fractions were dried under vacuum after the addition of 2 μl of 10% SDS to each aliquot. The dried residue was dissolved in 40 μl of non-reducing sample buffer, boiled for 3 min and then subjected to electrophoresis on a 12% polyacrylamide gel according to the procedure of Laemmli[12]. Gels were silver-stained by the method of Oakley *et al.*[13].

Protein sequencing was performed on an Applied Biosystems model 470A sequencer by using the chemicals and program supplied by the manufacturer. Phenylthiohydantoin (>PhNCS) amino acids were identified by reversed-phase HPLC, as described by March and Hopp[14]. The conditions used for *in situ* cyanogen bromide cleavage are described in the legend to Table II.

RESULTS

Purification of human IL-2

The initial steps in the purification of IL-2 from media conditioned by Jurkat-H33JA1 cells consisted of ammonium sulfate precipitation, followed by ion-exchange chromatography on carboxymethyl Sepharose and finally on diethylaminoethyl Sephacel. The volume of material at this stage of purification was usually around 100 ml with a protein concentration of 700 μg/ml.

This material in 2.5 m*M* Hepes, 20 m*M* sodium chloride, pH 7.4, was made 0.9 *M* in acetic acid and pumped directly onto a Whatman Protesil C_8 reversed-phase column that had been equilibrated in 0.9 *M* pyridine, 0.2 *M* acetic acid. A gradient of propanol in this buffer then eluted the proteins from the column. Under the conditions described, IL-2 was eluted by 48% propanol, 114 min after the gradient was started.

The fractions containing IL-2 were pooled and diluted 1:3 with 0.1% trifluo-

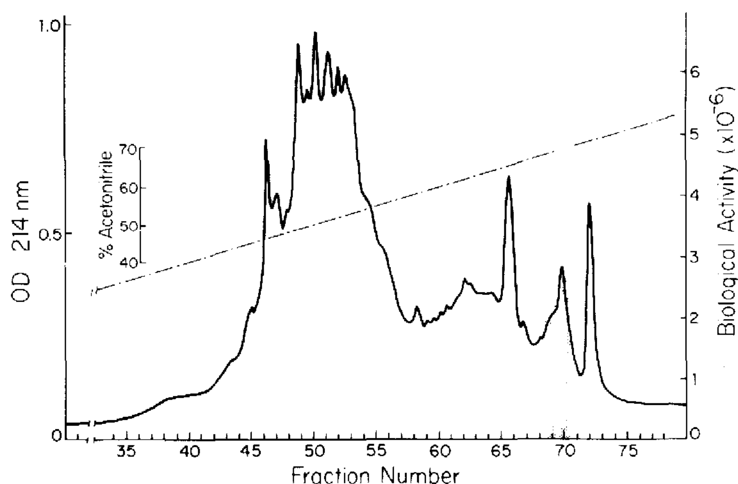

Fig. 1. IL-2 purification by reversed-phase HPLC. Solid line, absorbance at 214 nm. Hatched line, biological activity as measured in the CTLL assay. IL-2-containing fractions from the C_8 column in acetic acid–pyridine propanol were diluted 1:3 with 0.1% TFA and applied to a Waters C_{18} μBondapak column. A linear gradient from 0 95% solvent B (0.1% TFA in acetonitrile) at 1% per minute was initiated. One-min fractions were collected and aliquots from each fraction were diluted 1:1000 in Clicks medium (Altick Assoc., Madison, WI, U.S.A.), containing 10% fetal calf serum, and tested for activity in the CTLL assay.

roacetic acid (TFA). This solution was then pumped onto a Waters C_{18} μBondapak column. The loaded column was washed with 0.1% TFA until the absorbance at 214 nm dropped to baseline. At this point, a linear gradient of acetonitrile at 1% per min was established. As illustrated in Fig. 1, many peaks were resolved by this procedure. IL-2 activity was eluted in a sharp peak by 70% acetonitrile. Table I summarizes the purification scheme and reveals that the IL-2 at this stage had been purified 1 million fold, mostly as a result of the final two reversed-phase steps.

Aliquots of the fractions were analyzed by polyacrylamide gel electrophoresis and silver staining. As depicted in Fig. 2, the fractions containing IL-2 activity (No. 69 and No. 70) were composed primarily of two protein bands at molecular weights

TABLE I

IL-2 PURIFICATION

Fraction	*Specific activity (u/μg)*	*Yield (% of total units)*	*Purification fold*
Conditioned medium	2*	100	1
DEAE Sephacel	$3.20 \cdot 10^{2}$*	118**	160
Protesil C_8, Pyridine	$3.9 \cdot 10^{4}$*	123**	$2 \cdot 10^{4}$
Waters C_{18}, TFA	$2.0 \cdot 10^{6}$***	184**	$1 \cdot 10^{6}$

* Protein determined by the fluorescamine assay[24] with BSA as standard.

** The increase in yield is thought to be due to elimination of inhibitory factors.

*** Specific activity in the peak fraction, based on estimates from silver-stained gels and protein sequencer data.

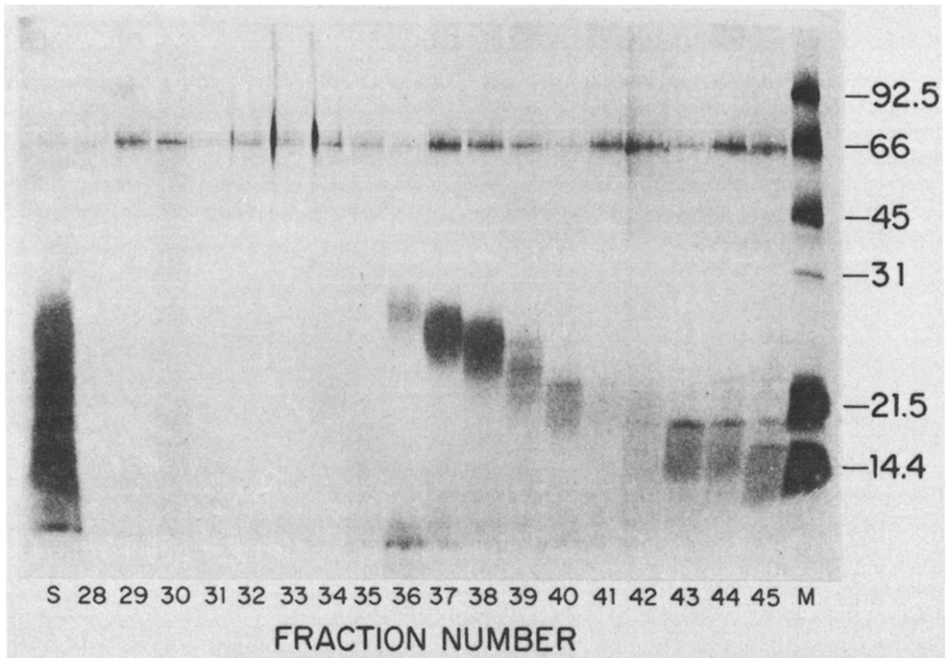

Fig. 2. Analysis of HPLC-purified IL-2 by polyacrylamide gel electrophoresis and silver-staining. S = starting material. Fraction number corresponds to fractions collected during the chromatogram depicted in Fig. 1. 20-μl aliquots of each fraction were dried with 200 μg SDS. The dried residue was dissolved in 40 μl of non-reducing sample buffer, boiled for 3 min and then subjected to electrophoresis on a 12% polyacrylamide gel according to the procedure of Laemmli[12]. Gels were silver-stained by the method of Oakley *et al.*[13].

of 15,000 and 16,000. The peak fraction (50 pmole) was dried to a small volume and applied to the sample filter of the protein sequencer.

N-terminal sequencing was performed for 13 cycles, but without detectable sequence, suggesting the presence of a blocked N-terminal. The filter was removed and cyanogen bromide cleavage was performed *in situ*. After the cyanogen bromide reaction, the filter was dried under vacuum and placed back in the sequencer. The resulting cyanogen bromide fingerprint of IL-2 is presented in Table II. The observed residues were listed according to the residues one would predict from the published DNA sequence for IL-2[15]. They matched exactly, confirming the DNA sequence at the protein level.

Purification of CSF-2α

The initial steps in the purification of CSF-2α were very similar to those followed for IL-2. CSF-2α was precipitated from medium conditioned by LBRM-33 cells by ammonium sulfate and fractionated by ion-exchange chromatography on DEAE Sephacel followed by chromatography on SP Sephadex. The material at this stage was usually in a volume between 100 and 300 ml and at a protein concentration of 20 to 40 μg/ml.

This material in 10 m*M* sodium citrate, 200 m*M* sodium chloride was made

TABLE II

IN SITU CYANOGEN BROMIDE CLEAVAGE OF HUMAN IL-2

N-terminal sequencing was performed for 13 cycles. The filter was then removed from the sequencer and saturated with 30 μl of cyanogen bromide solution (5 mg cyanogen bromide/100 μl 70% formic acid). The filter along with the tube containing the remaining cyanogen bromide solution was then sealed in a scintillation vial and incubated overnight at room temperature. The filter was then dried under vacuum and placed back in the sequencer. The Applied Biosystems Model 470A sequencer was operated with the chemicals and program supplied by the manufacturer. >PhNCS amino acids were identified by reversed-phase HPLC as described by March and Hopp[14].

Cycle	*(Met^{43}) Residue 1*	*(Met^{59}) Residue 2*	*(Met^{66}) Residue 3*	*(Met^{124}) Residue 4*
1	Ile (30.46)*	Leu (29.92)	Pro (14.72)	–**
2	Leu (44.37)	Thr (10.00)	Lys (12.00)	Glu (22.00)
3	Asn (29.00)	Phe (10.00)	Lys (15.00)	Tyr (30.00)
4	Gly (20.00)	Lys (5.00)	Ala (50.00)***	Ala***
5	Ile (16.00)	Phe (4.97)	Thr (11.00)	Asp (20.04)
6	Asn (15.00)	Tyr (9.07)	Glu (30.00)***	Glu***
7	Asn (17.13)	–	Leu (10.09)	Thr (6.74)
8	Tyr (11.49)	–	Lys (11.36)	Ala (12.46)
9	Lys (12.32)	–	His (6.02)	Thr (5.79)
10	Asn (5.16)	–	Leu (7.47)	Ile (4.28)
11	Pro (4.00)	–	Gln (4.26)	Val (3.65)
12	Lys (3.01)	–	–**	Glu (2.98)
13	Leu (8.76)***	–	Leu***	Phe (1.79)
14	Thr (2.07)	–	Glu (3.28)	Leu (2.54)
15	Arg (0.96)	–	Glu (3.41)	Asn (1.59)
16	–	–	Glu (2.98)	Arg (0.97)
17	–	–	Leu (1.13)	Trp (0.85)
18	–	–	Lys (0.91)	Ile (1.11)

* Number in parenthesis are pmoles of >PhNCS amino acid.

** Cys, the expected residue for this position, was not identified because sulfydryl groups were not modified before sequencing.

*** Expected residue for more than one peptide in this cycle.

TABLE III

CSF-2α PURIFICATION

n.d. = not determined.

Fraction	*Specific activity (u/μg)*	*Yield (% of total units)*	*Purification fold*
Conditioned medium	4.4*	100	1
$(NH_4)_2SO_4$ precipitate	4.37 · 10*	64	9.9
DEAE Sephacel	1.17 · 10^2*	47	26.5
SP Sephadex	7.00 · 10^3*	9	1.6 · 10^3
Waters C_{18}, TFA	n.d.	11	n.d.
Waters C_{18}, pyridine	1.06 · 10^7**	10	2.4 · 10^6

* Protein determined by the BioRad Protein Assay (BioRad, Richmond, CA, U.S.A.) with BSA as standard.

** Specific activity in the peak fraction based on estimates from silver stained gels.

0.1% in TFA and pumped onto a Waters C_{18} μBondapak reversed-phase column previously equilibrated in 0.1% TFA. Proteins were eluted by a linear gradient of acetonitrile. Under these conditions, CSF-2α was eluted in a sharp peak by 48% acetonitrile, 48 min after the gradient had been started.

The fractions containing CSF-2α activity were pooled and diluted 1:3 with 0.9 *M* acetic acid, 0.2 *M* pyridine and applied the same C_{18} column previously equilibrated with the pyridine–acetate buffer. A gradient of *n*-propanol was established to elute the protein from the column. CSF-2α was eluted by 28% propanol 54 min after the gradient had been started. Table III summarizes the purification procedure and indicates that CSF-2α was purified 2 million fold. It is important to note that although overall recoveries of CSF-2α were lower than the recoveries with Il-2, the recovery of CSF-2α at the HPLC steps was essentially 100%.

Aliquots of the final HPLC were analyzed by polyacrylamide gel electrophoresis (Fig. 3). Fraction 37 contained the bulk of the CSF-2α activity and consisted of a single major band with molecular weight of 24,500. We estimate the specific activity of the final material to be $1.1 \cdot 10^7$ u/μg.

DISCUSSION

Reversed-phase HPLC of proteins and peptides is influenced by a number of

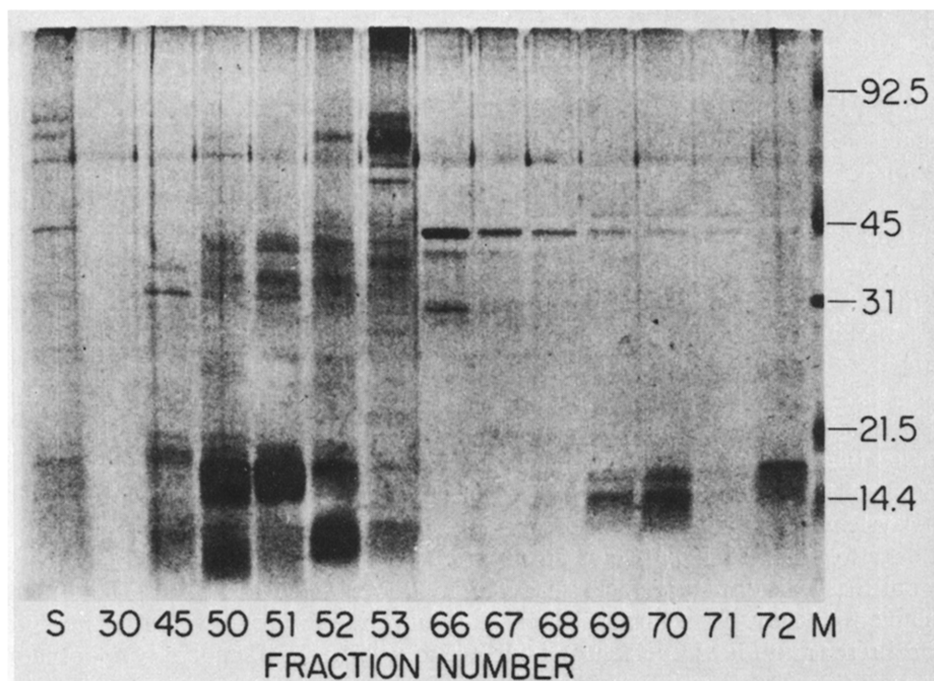

Fig. 3. Analysis of HPLC purified CSF-2α by polyacrylamide gel electrophoresis and silver-staining. The fraction containing CSF-2α from HPLC in TFA–acetonitrile was diluted 1:3 with 0.9 *M* acetic acid, 0.2 *M* pyridine and applied to a C_{18} μBondapak column equilibrated in solvent A. A gradient of solvent B went from 0 to 20% B in 10 min and from 20% to 84% B in 110 min at a flow-rate of 0.7 ml/min. 1.5-min fractions were collected from which 40-μl aliquots were taken for analysis on the gel. Fraction 37 contained the bulk of CSF-2α activity and consisted of a single diffuse band of molecular weight 24,500.

variables, including the nature of the stationary phase, the solvent and the pH of the mobile phase. In designing the purification scheme for IL-2 we capitalized upon the notion that alterations in the mobile phase can have dramatic effects on the separation of proteins by reversed-phase chromatography[16,17,18]. We found that chromatography of IL-2 in pyridine–acetate–propanol at pH 4.0, followed by chromatography in TFA–acetonitrile at pH 2.0, yielded material that had been purified 1 million fold over crude conditioned medium.

The peak fraction from the final chromatographic step contained two protein bands with molecular weights of 15,000 and 16,000. Protein sequencing of this fraction was attempted in order to identify these two bands. However, as no sequence was detected, the presence of a blocked N-terminus is suggested. We were able to perform *in situ* cyanogen bromide cleavage and thus were able to produce a cyanogen bromide fingerprint of this fraction that established its identity as IL-2. The lack of any other major residues in either N-terminal sequencing or after cyanogen bromide cleavage suggested that both bands seen on the polyacrylamide gel (Fig. 2) were IL-2. Henderson *et al.*[6] recently described four molecular forms for Gibbon ape IL-2. Three of them differed in isoelectric point but ran at a molecular weight of 16,000. The fourth ran at a molecular weight of 15,000. Each was biologically active, reinforcing the suggestion that the two bands we observe for human IL-2 are different molecular weight forms of this molecule. It is possible that the different molecular weight forms of IL-2 are due to differences in glycosylation.

The procedure described for *in situ* cyanogen bromide cleavage is a valuable technique that permits the extraction of the maximum amount of information from any given sample. Work with naturally occurring lymphokines will rarely be above the picomole level, and techniques that permit analysis at this level will prove indispensible.

The experience gained in the purification of IL-2 was applied to the purification of an unrelated lymphokine, murine CSF-2α. CSF-2α is one of three colony stimulating activities (CSF-2α, CSF-2β and CSF-2γ) that is elaborated by LBRM-33 cells[10]. In this case we used the same reversed-phase column (C_{18} μBondapak) and by changing the mobile phase system were able to purify this material 2 million fold to apparent homogeneity.

CSF-2α is similar biochemically and functionally to a number of other lymphokines in addition to CSF-2β and CSF-2γ that include P-factor[19], mast-cell growth factor[20], IL-3[21,22] and hematopoietic cell growth factor[5,12,23]. These factors however are difficult to compare because the source of factor, preparative procedures and assay systems are all different. Conclusions regarding the relationship between them will have to wait until each factor has been purified to homogeneity and sequenced or until the genes for these factors have been cloned and recombinant factors are available for study. Sequencing of CSF-2α is currently in progress and we hope to report presently on how it relates on a molecular level to this family of lymphokines.

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