Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 640–644, February 1979
Biochemistry

Human leukocyte interferon: Production, purification to homogeneity, and initial characterization

(high-performance liquid chromatography/protein fractionation/fluorescence detection/antiviral agent)

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Communicated by Sidney Udenfriend, November 8, 1978

ABSTRACT **A method of fractionating proteins by high-performance liquid partition chromatography has been developed and used for isolation and purification to homogeneity of one of the species of human leukocyte interferon. The homogeneous interferon exhibited a sharp peak on high-performance liquid chromatography and a single narrow band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. Extraction of the gel gave a single sharp peak of antiviral activity coinciding with the protein band. The specific activity of pure interferon was found to be 2–4 × 10^8 units/mg, based on amino acid analysis. The molecular weight is 17,500–18,000.**

Interferon was described as an antiviral activity by Isaacs *et al.* (1, 2) and independently by Nagano and Kojima (3). Since that time, a vast literature on interferon has accumulated (see ref. 4 for reviews). This body of data provides evidence that, even within a single species, several biologically, physically, and chemically distinct antiviral activities have properties that classify each of them as an interferon. There is even evidence for more than one structural gene for interferons in human cells (5–7). Although much information is available on the physical and chemical properties of interferons, no clear definitive structure has emerged. The lack of reliable data on chemical composition has been due to the failure to obtain significant amounts of any pure interferon for analysis despite many substantial programs directed toward purification. Numerous attempts have been made to purify an interferon by conventional protein fractionation techniques (8–15) as well as by affinity chromatography with antibodies (10–12, 16, 17), lectins (18, 19), polynucleotides (12), and numerous other small ligands (19–21). Indeed, it has been reported that human fibroblast interferon (9, 15) and mouse L-cell interferon (12, 14) have been purified to apparent homogeneity. However, no chemical composition or any characterization of these components has yet been provided.

In the past few years, analysis and detection of amino acids, peptides, and proteins at the picomole level by fluorescence has been developed by Udenfriend and coworkers (22–24). More recently, this technique has been combined with high-performance liquid chromatography for the rapid fractionation and detection of peptides up to a molecular weight of 12,000 (25, 26). The techniques have now been further modified and applied to the purification of human leukocyte interferon. The methods offer a combination of high resolution and high sensitivity that is essential for the purification of proteins, such as interferon, that are present in small quantity in cell extracts or growth media. These methods have been utilized along with novel procedures for interferon production and assay to purify human leukocyte interferon to homogeneity in amounts sufficient for chemical characterization.

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EXPERIMENTAL PROCEDURE

Interferon Production and Assay. Human leukocytes were isolated from the buffy coat fraction of whole blood from normal donors and used for the induction of interferon with Newcastle disease virus (27, 28). Casein was substituted for serum (29) to provide a simpler milieu from which to isolate the interferon. The procedures used for induction were performed as reported (27–29) with modifications that will be described in detail elsewhere. Interferon titers were determined by a cytopathic effect-inhibition assay that was modified so that the entire assay could be performed within 16 hr. Details of this assay will be published elsewhere. All interferon titers are expressed in terms of reference units/ml, calibrated against the reference standard for human leukocyte interferon (G-023-901-527). We are grateful to G. J. Galasso, J. K. Dunnick, and the National Institute of Allergy and Infectious Diseases (National Institutes of Health) for a supply of the reference standards for human interferon.

Detection of Amino Acids, Peptides, and Proteins by Fluorescence. Amino acid analyses were performed on a fluorescamine analyzer as described (23). The high-pressure liquid chromatography system for peptides has been described (25). An automated fluorescence detection system was used for monitoring peptides in column effluents (24). High-performance liquid chromatography columns were obtained from EM Laboratories (Elmsford, NY). Pyridine, acetic acid, and formic acid were distilled twice over ninhydrin. All column buffers contained thiodiglycol (0.01%). Polypropylene tubes and laboratory ware were used for fractions containing interferon. Proteins were assayed by injection of samples into the fluorescamine peptide/protein monitoring system with bovine serum albumin as a standard. This method gave excellent reproducibility and sensitivity with nanogram quantities.

Slab Gel Polyacrylamide Electrophoresis. Slab gel polyacrylamide electrophoresis was performed on 12.5% polyacrylamide gels in a Tris·glycine (pH 8.4) buffer in the presence of 0.1% sodium dodecyl sulfate ($NaDodSO_4$) as described (30). Protein standards as well as interferon were incubated in 1% (wt/vol) $NaDodSO_4$ and 2% (vol/vol) 2-mercaptoethanol for 1 hr at room temperature prior to electrophoresis. Incubation of protein markers under these conditions at higher temperature did not cause any change in mobility.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

RESULTS

Production of Interferon. Interferon was produced by incubation of human leukocytes (10^7 cells per ml) with Newcastle disease virus (15 hemagglutination units/ml) in a serum-free minimal essential medium containing casein (10 mg/ml) for 16 hr. Initial pilot experiments yielded titers of 10,000–20,000 units/ml. On scale up, an average titer of 5000 units/ml was obtained. Leukocytes from 50–100 buffy coats were processed weekly, yielding a total of about 10^9 units over a 9-month period.

Concentration and Initial Fractionation of Interferon. Unless otherwise specified, these procedures were performed at 0–4°C. At the end of the incubation, cells and debris were removed by low-speed centrifugation (15 min, 500 × *g*). Casein was precipitated by acidification with HCl to pH 4.0. After 2 hr, the mixture was centrifuged (10 min, 12,000 × *g*) and the pellet was discarded. The supernatant (10 liters) was adjusted to 1.5% (wt/vol) trichloroacetic acid. After 1 hr, the precipitate was collected by centrifugation (10 min, 12,000 × *g*) and redissolved in 50 ml of 0.1 M $NaHCO_3$. Triton X-100 (0.5 g) was added and then acetic acid (1.5 ml) was added dropwise with stirring. The mixture was stored at 0°C for 1 hr and then for 16 hr at −20°C. It was then thawed and centrifuged (10 min, 17,000 × *g*). The pellet was discarded and the supernatant was adjusted to 4% trichloroacetic acid. After 1 hr, the mixture was centrifuged (10 min, 12,000 × *g*). The precipitate was collected and redissolved in 5 ml of 0.5 M $NaHCO_3$.

Gel Filtration. Urea (1.5 g) was added to the interferon concentrate and the solution was applied to a column of Sephadex G-100 fine (2.6 × 90 cm). The column was eluted with 4 M urea/0.1 M sodium acetate, pH 7.5, at room temperature at a flow rate of 0.5 ml/min. Fractions of 12.5 ml were collected. Interferon was eluted in fractions 19–23 (Fig. 1).

High-Performance Liquid Chromatography. Fractions 19–23 were combined and applied directly through the pump to a Lichrosorb RP-8 column (10 μm; 4.6 × 250 mm). The column was washed with 1 M sodium acetate buffer (pH 7.5) and then eluted with a 1-propanol gradient [1 hr, 0–20%; 3 hr, 20–40% (vol/vol)] in the same buffer at a flow rate of 0.25 ml/min. Fractions of 0.75 ml were collected. Interferon was eluted in fractions 23–40 (Fig. 2*A*). Fractions 27–33, containing most of the interferon activity, were combined, 1-propanol was added to a final concentration of 80% (vol/vol), and the solution was applied through the pump to a Lichrosorb diol column (10 μm; 4.6 × 250 mm) previously equilibrated with a solution of 0.1 M sodium acetate containing 80% (vol/vol) 1-propanol. The column was then eluted with a linear 4-hr gradient of 72.5–50% 1-propanol in 0.1 M sodium acetate at a flow rate of 0.25 ml/min. Fractions of 0.75 ml were collected. Interferon activity was eluted as three distinct major peaks that varied quantitatively from one preparation to another (Fig. 2*B*). For purposes of discussion, the peaks are labeled α, β, and γ according to their order of elution. The fractions comprising each peak were pooled separately and purified individually through subsequent steps. Because peak γ was present in high abundance and appeared to be better resolved from other components, it was selected for further purification. Fractions 54–56, comprising peak γ from the diol column, were pooled and 1-propanol was removed by two extractions with an equal volume of hexane. Traces of hexane were removed under a stream of nitrogen. Pyridine and formic acid were added to final concentrations

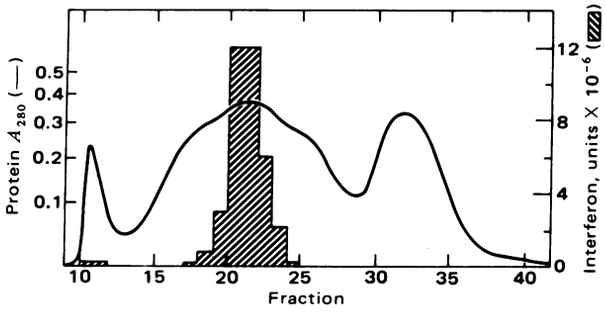

FIG. 1. Chromatography of crude leukocyte interferon on a Sephadex G-100 column in 4 M urea/0.1 M sodium acetate, pH 7.5. See text for details.

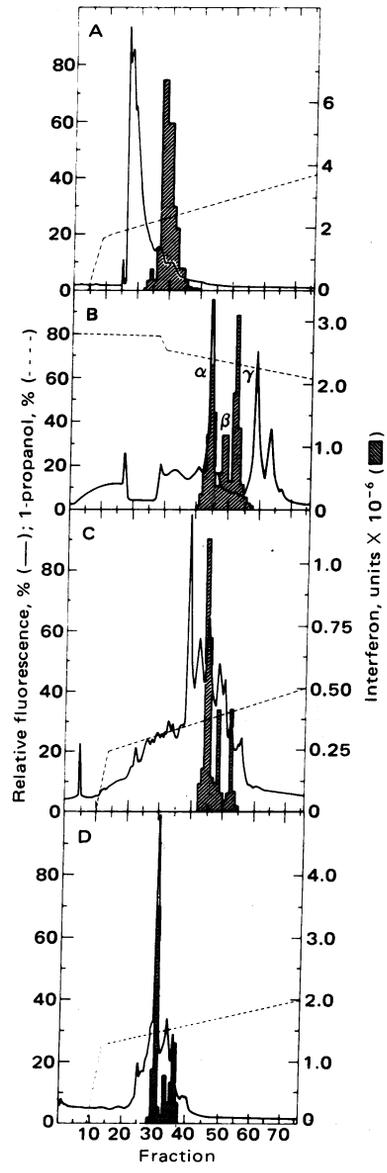

FIG. 2. High-performance liquid chromatography of interferon. (*A*) Chromatography on Lichrosorb RP-8 at pH 7.5. The fluorometer scale was set to 100 and 2% of the column effluent was directed to the fluorescamine monitoring system. (*B*) Chromatography on Lichrosorb diol at pH 7.5. The fluorometer scale was 30 and 2% of the column effluent was directed to the fluorescamine monitoring system. (*C*) Chromatography on Lichrosorb RP-8 at pH 4.0. The fluorometer scale was 1 and 5% of the column effluent was directed to the fluorescamine monitoring system. (*D*) Rechromatography on Lichrosorb RP-8. The conditions were similar to those of step *C*. Several preparations carried through step *C* were pooled (13 × 10^6 units) and applied to the last column. The gradations on the abscissa correspond to the end of the fractions.

Table 1. Purification of human leukocyte interferon

Step	Units recovered $\times 10^{-6}$	Protein recovered, mg	Relative specific activity, units/mg	Degree of purification	Recovery range per step, %
1. Incubation medium	50	10,000	5×10^3	1	—
2. pH 4 supernatant	50	2,000	2.5×10^4	5	100
3. 1.5% trichloroacetic acid precipitate	40	1,000	4×10^4	8	80–100
4. Triton X-100/acetic acid supernatant	40	250	1.6×10^5	32	70–100
5. 4% trichloroacetic acid precipitate	35	175	2×10^5	40	80–90
6. Sephadex G-100	32	57	5.6×10^5	112	70–90
7. Lichrosorb RP-8 (pH 7.5)	28	11	2.5×10^6	500	80–100
8. Lichrosorb diol					
Peak α	11	1.1	1×10^7	5,000	70–90
Peak β	2.5	ND	ND	ND	
Peak γ	12.5	0.21	6×10^7	12,000	
9. Lichrosorb RP-8 (pH 4)	1.6	0.0064	3×10^8	60,000	40–60
10. Lichrosorb RP-8 (pH 4)	8.2	0.021	4×10^8	80,000	40–60

For determination of protein recovered in each fraction, bovine serum albumin was used as a standard. The absolute specific activity determined by amino acid analysis of the homogeneous peak of step 10 was found to be 2–4 $\times 10^8$ units/mg (see text). Step 10 was performed on pooled material from several preparations. ND, not determined.

of 1 M and 2 M, respectively, and the solution was applied to a Lichrosorb RP-8 column (10 μm; 4.6 $\times$ 250 mm) previously equilibrated with 1 M pyridine and 2 M formic acid (pH 4.0). The column was eluted with a linear 20–40% 1-propanol gradient in the 1 M pyridine/formate buffer in 3 hr at a flow rate of 0.2 ml/min. Fractions of 0.6 ml were collected. The major peak of activity coincided with a protein peak (Fig. 2*C*). Fractions 45 and 46 comprising this peak were combined and rechromatographed under similar conditions (Fig. 2*D*). Interferon was eluted in fraction 31. The specific activity of this fraction was calculated to be 4 $\times 10^8$ units/mg in relation to bovine serum albumin. This material was used for further characterization. The fluorescence profiles of the high-performance steps were so remarkably reproducible that they provided a continual fingerprint of the entire procedure.

The results of the purification are summarized in Table 1. The overall purification starting with the incubation medium to the second RP-8 column was 60,000- to 80,000-fold. The cumulative yield from step 1 through the diol step ranged from 30 to 50%. Beyond this step, each of the three peaks of interferon was purified separately.

Table 2. Amino acid composition of human leukocyte interferon

Amino acid	Residues
Asx	15.2 ± 1.2
Thr*	7.5 ± 0.5
Ser*	8.0 ± 0.5
Glx	24.0 ± 0.6
Pro	6.3 ± 0.3
Gly	5.5 ± 0.5
Ala	8.2 ± 0.2
Cys (total)	3.3 ± 0.7
1/2 Cystine†	1.8 ± 0.2
Cysteine†	1.5 ± 0.5
Val	7.8 ± 0.2
Met	3.9 ± 0.2
Ile	8.9 ± 0.4
Leu	21.8 ± 1.3
Tyr	5.1 ± 0.2
Phe	9.1 ± 0.3
His	3.3 ± 0.4
Lys	11.6 ± 0.5
Arg	7.3 ± 0.5
Trp‡	0.7 ± 0.1

* Corrected to time 0.
† Measured after carboxymethylation of native interferon.
‡ Measured after hydrolysis in 6 M HCl/4% thioglycolic acid.

Polyacrylamide Gel Electrophoresis. Samples of interferon (1.5 $\times 10^5$ units) were incubated in $NaDodSO_4$ and 2-mercaptoethanol and then applied to a slab gel. After electrophoresis, a single sharp band was obtained upon staining with Coomassie blue (Fig. 3). The apparent molecular weight was estimated to be 17,500 in comparison with standard proteins. The gel was then cut into 1-mm slices. Each slice was homogenized in 0.4 ml of 0.5 M $NaHCO_3$/0.1% $NaDodSO_4$ and assayed for interferon activity. A single peak of antiviral activity was obtained coinciding with the single protein band. No other peak of activity was observed. Details of these latter experiments are described elsewhere (31).

Amino Acid Analysis. Amino acid analysis of homogeneous human leukocyte interferon (peak γ) was performed with the fluorescamine amino acid analyzer on 0.5 to 1-μg samples of native and *S*-carboxymethylated interferon. For measurement of the cysteine/cystine ratio, native interferon was carboxymethylated and then hydrolyzed in 6 M HCl under reducing conditions (0.1% thioglycolic acid). Under these conditions, cysteine is measured as *S*-carboxymethylcysteine whereas cystine is measured as free cysteine. Amino acid analyses are summarized in Table 2. The specific activity based on amino acid content was found to be 2–4 $\times 10^8$ units/mg.

DISCUSSION

We chose to induce interferon biosynthesis in a serum-free medium containing casein. The omission of serum simplified the purification, and the insolubility of casein at low pH enabled us to obtain high initial specific activity. Crude interferon contains proteins secreted by the leukocytes, some proteins from disrupted cells, and fragments of casein. Like many other purification schemes, the first stages were selective precipitations to remove the bulk of the proteins and to reduce the volume. Neither ammonium sulfate nor organic solvent fractionation provided any purification because interferon tends to coprecipitate with other proteins. We have studied this phenomenon and found that detergents can prevent it. Thus, we concluded that coprecipitation is the result of hydrophobic interactions. Fractionation under acidic conditions in the presence of Triton X-100 gave the best results.

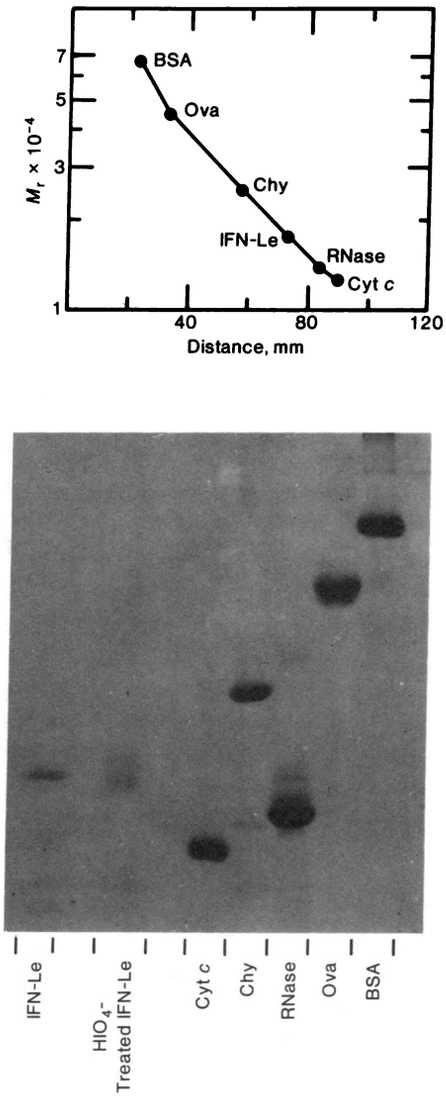

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of human leukocyte interferon (IFN-Le). The markers used were: bovine serum albumin (BSA), ovalbumin (Ova), chymotrypsinogen (Chy), ribonuclease (RNase), cytochrome *c* (Cyt *c*).

Gel filtration at various pH values produced a broad distribution of activity and resulted in poor recovery (40–50%). Only under denaturing conditions (4 M urea) did gel filtration provide an excellent yield and a relatively narrow distribution of activity. Ion exchange chromatography, hydrophobic chromatography on octyl-Sepharose, and chromatography on poly(U)-Sepharose were found to be unsatisfactory.

Several papers have described high-performance liquid chromatography of proteins, mainly on ion exchange and size exclusion columns (32, 33). However, these systems are either not commercially available or have a low capacity. Our previous studies with Lichrosorb RP-18 (octadecyl groups bound to silica microparticles) have clearly shown the high resolution of reverse-phase partition chromatography with respect to large peptides (24, 26). Use of Lichrosorb RP-8 (octyl groups bound to porous silica microparticles) rather than RP-18 permits the fractionation of proteins. We consider this application of Lichrosorb RP-8 columns to protein fractionation a major factor in the success of the purification procedure (see Table 1). Lichrosorb diol is chemically similar to glycophase resins that have been used for exclusion chromatography of proteins (32). In our study, Lichrosorb diol was introduced as a support for normal partition chromatography of proteins. High recoveries of interferon activity were obtained in each chromatographic step.

Bioassay of the fractions from NaDodSO$_4$/polyacrylamide gel electrophoresis of partially purified preparations of human leukocyte interferon have yielded two major bands, one of molecular weight 15,000–18,000 and the other, 21,000 (10, 11, 34, 35). In this study, NaDodSO$_4$/polyacrylamide gel electrophoresis was performed on homogeneous interferon reduced with 2-mercaptoethanol. Both bioassay and the protein band corresponded to a molecular weight of 17,500. When a sample of interferon was treated with periodic acid (0.01 M, 16 hr, pH 4, 25°C, in the dark) and subjected to electrophoresis, it was converted to a broad doublet but no appreciable reduction in molecular weight was observed. If this species of interferon is a glycoprotein, then the degree of glycosylation is not great or the glycosidic residues have only a small effect on mobility.

On the basis of cystine content, a minimal molecular weight of 18,000 was calculated. This value is in good agreement with that obtained on gel electrophoresis. Thus, interferon has one disulfide bridge and one or two free sulfhydryl groups.

It should be noted that only one species of interferon was purified to homogeneity and characterized. However, other species of interferon were observed (Fig. 2*b*), and these require purification and characterization. The precise relationship of these species to one another, to the secreted material, and to the primary gene product is of major interest.

Interferons have been reported to have a large number of effects such as antiviral, antitumor, growth inhibition, and immunosuppression (see ref. 4 for reviews). Because most of those assays were performed with relatively crude preparations, less than 1% of which was human interferon, the results could have been due to components other than interferon. The availability of pure interferon now makes it possible to resolve these uncertainties.

We are most grateful to Dr. Sidney Udenfriend for his enthusiastic support and valuable guidance throughout this study. We also thank Dr. Stanley Stein for making available unique instrumentation he developed for picomole assay of proteins and amino acids. We thank Dr. Kenneth Woods for helpful discussions. Special thanks are owed to Mr. Larry Brink for performing the exacting amino acid analyses, to Mr. Mitchell Gross for performing the slab gel electrophoresis, and to Mr. Donald Glover for assistance in interferon production.

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