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RAPID PURIFICATION OF LEUKOCYTE INTERFERONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Many species of interferon have been identified and purified from human leukocytes, and genes for these interferons have been cloned in bacterial systems. However, it is still unclear as to how the expression of each of the different leukocyte interferons is regulated and how they function in the natural system. In preparation for a study of the *in vivo* role of individual leukocyte interferons we have developed a rapid and convenient high-performance liquid chromatographic method for purifying several species of human leukocyte interferon. Interferon produced by the induction of leukocytes (obtained from buffy coats of normal donors) with Sendai virus and partially purified by ethanol fractionation was further purified by size-exclusion high-performance liquid chromatography (on two Waters I-125 columns) in 0.05 *M* sodium phosphate buffer, pH 6, containing 0.2 *M* sodium chloride. The flow-rate was 2 ml/min and interferon activity (antiviral) was eluted from the columns in less than 15 min in a single fraction well resolved from high-molecular-weight contaminants. This material was then purified by reversed-phase high-performance liquid chromatography on a wide-pore diphenyl column (Whatman Protesil 300) with a gradient system at pH 2.4, consisting of (A) 0.05 *M* KH_2PO_4–methoxyethanol (19:1) and (B) 1-propanol–methoxyethanol (19:1). Several peaks of interferon activity were distinguishable in this system. Analysis of the pooled peaks of interferon activity by SDS polyacrylamide gel electrophoresis confirmed the presence of more than one form of leukocyte interferon. The specific activity of the purified leukocyte interferons was *ca.* 10^8 units/mg protein.

INTRODUCTION

Interferons are proteins exhibiting numerous biological properties, including the ability to inhibit viral replication in cells, the ability to inhibit cell proliferation, and the ability to stimulate natural killer cell activity. Three classes of interferons have been distinguished biochemically and immunologically and are identified as leukocyte, fibroblast, and immune interferons. Leukocyte interferon has been shown to consist of a family of at least twelve related proteins which possibly differ in their biological activities[1]. Most of these interferon "subspecies" have been identified by

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the analysis of human chromosomal DNA although several have been produced and purified from human leukocytes[2].

In preparation for a study of the regulation and expression of individual leukocyte interferons and the role of each *in vivo*, we have developed a rapid and convenient method for purifying several species of leukocyte interferon by high-performance liquid chromatography (HPLC). The method described here utilizes several commercially available (wide-pore) HPLC columns designed specifically for protein separations.

EXPERIMENTAL

Apparatus and materials

The liquid chromatograph consisted of a Model 6000A pump, a Type U6K injector, and a Model 450 variable-wavelength UV detector, all products of Waters Assoc., Milford, MA, U.S.A. The Protesil 300 diphenyl column (250 × 4.6 mm I.D., 10 μm) and the Protesil 300 C_8 column (250 × 4.6 mm I.D., 10 μm) were obtained from Whatman, Clifton, NJ, U.S.A., and the Bakerbond Wide Pore C_{18} column (250 × 4.5 mm I.D., 10 μm) from J. T. Baker, Phillipsburg, NJ, U.S.A. The Waters I-125 columns (300 × 7.8 mm I.D., 10 μm) were purchased from Waters Assoc. The 1-propanol and 2-methoxyethanol were Burdick & Jackson (Muskegon, MI, U.S.A.) HPLC-grade solvents. HPLC-grade water was obtained from Mallinckrodt, Paris, KY, U.S.A. All other chemicals were reagent or analytical grade.

Protein determination

Protein concentration was measured by either fluorometric assay[3] or by the method of Lowry[4] using human serum albumin supplied by Alpha Therapeutic Corporation as a standard.

Interferon assay

Interferon titers of purified preparations of leukocyte interferon were determined by a modification of the plaque reduction assay described by Matsuo *et al.*[5] using human amnionic FL cells challenged by vesicular stomatitis virus (VSV). For rapid (20 h) assay of column fractions, the interferon titer was determined by the inhibition of the cytopathic effect of VSV on human amnionic FL cells in a modification of the assay described by Rubinstein *et al.*[6]. All results are expressed in International Reference Units according to the National Institutes of Health Standard, G-023-901-527.

Interferon production and partial purification

Crude human leukocyte interferon was produced by the induction of leukocytes (obtained from buffy coats of normal donors) with Sendai virus and then partially purified by ethanol fractionation as described by Cantell and Hirvonen[7]. Partial purification generally resulted in a greater than 100-fold increase in purity (over crude interferon) and a 70% recovery of original interferon activity. The minimum specific activity of partially purified leukocyte interferon was 10^6 units/mg protein.

RESULTS AND DISCUSSION

Leukocyte interferon was purified several thousand-fold in high yield by a combination of conventional chromatography and HPLC. Partial separation of interferon activity from high-molecular-weight contaminants still present after ethanol fractionation was accomplished by size-exclusion HPLC on two Waters I-125 columns (Fig. 1). Interferon activity was found to be eluted from these columns with a distribution coefficient (K_d) between 0.49 and 0.70 making it possible to collect only a single column fraction eluted between these K_d values which could then be concentrated and assayed. This fraction generally contained *ca.* 50–60% of the applied interferon activity. A purification of 4–7-fold was routinely obtains by this step, but because resolution was impaired when more than 3 mg of protein were applied, several successive injections were normally required to purify larger amounts of material by this procedure.

Chromatography of this purified interferon on a Whatman Protesil 300 diphenyl column yielded three distinct peaks of interferon activity (Fig. 2). These peaks most likely represent three classes of interferon, each composed of one or more

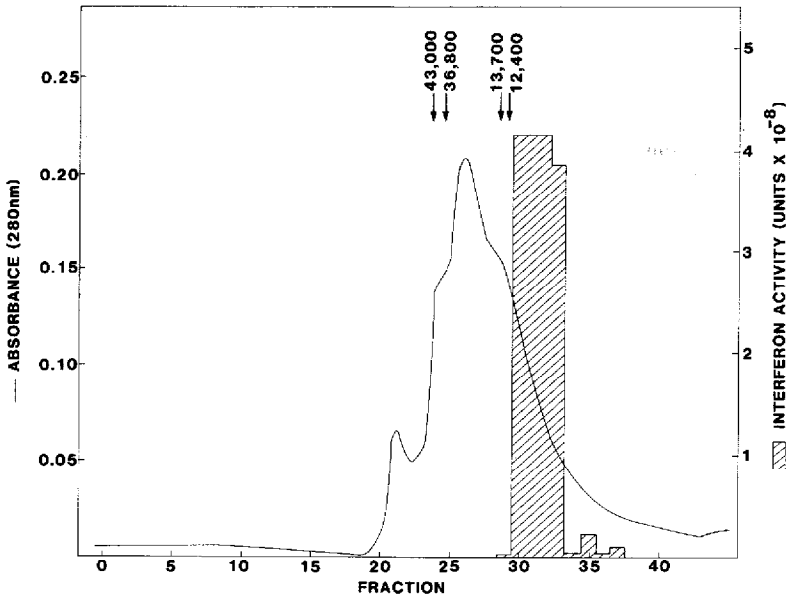

Fig. 1. Purification of leukocyte interferons by size-exclusion HPLC. Approximately 3 mg of human leukocyte interferon that had been partially purified by ethanol fractionation was chromatographed on two Waters I-125 columns connected in tandem and eluted with 0.05 *M* sodium phosphate buffer (pH 6.0) containing 0.2 *M* sodium chloride at a flow-rate of 2 ml/min. The absorbance was monitored continually at 280 nm with a Waters Model 450 spectrophotometer. Each fraction was collected for 0.3 min and all fractions eluted after the void volume were ssayed for interferon activity (inhibition of viral cytopathic effect). Arrows at the top of the figure indicate positions at which protein standards of the indicated molecular weight are eluted from the columns under identical conditions. Protein standards injected were ovalbumin, β-lactoglobulin A, ribonuclease A, and cytochrome *c* (horse heart). Catalase (mol.wt. 232,000) and guanosine (mol.wt. 283) were used to determine the void volume and the total non-matrix volume, respectively.

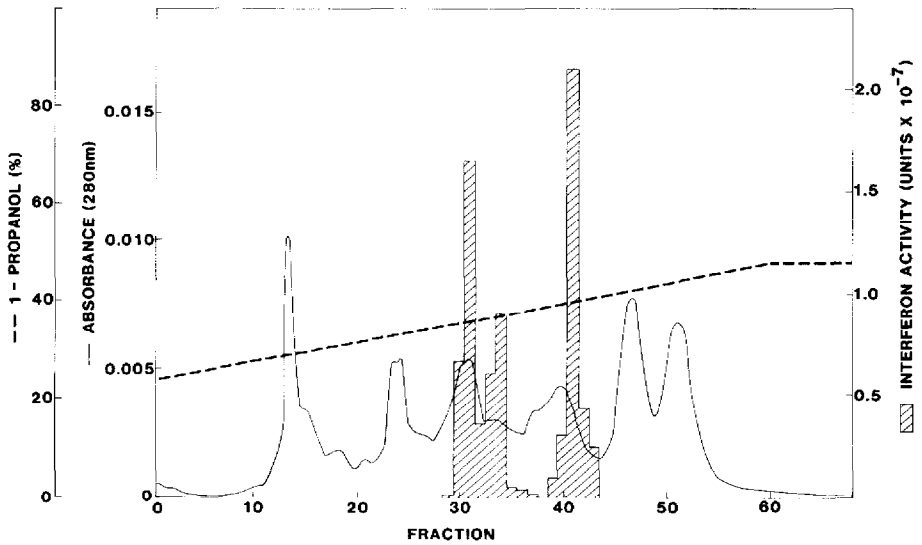

Fig. 2. HPLC purification of leukocyte interferons on a wide-pore diphenyl column. Approximately 1.8 mg of leukocyte interferon which had been purified by ethanol fractionation and size-exclusion HPLC (Fig. 1), was applied to a Whatman Protesil 300 diphenyl column that had previously been equilibrated with a mobile phase consisting of 25% Solvent B (1-propanol–2-methoxyethanol, 19:1, pH 2.4) and 75% Solvent A (0.05 *M* KH_2PO_4–2-methoxyethanol, 19:1, pH 2.4). The column was washed for several minutes with the same mobile phase, followed by elution of interferon activity with a linear gradient from 25 to 50% Solvent B in Solvent A. Gradient elution was accomplished in 1 h at a flow-rate of 0.8 ml/min. Absorbance was monitored continually at 280 nm using a Waters Model 450 spectrophotometer and fractions of 0.8 ml were collected and assayed for interferon activity (inhibition of viral cytopathic effect).

leukocyte interferon subspecies and differing in relative hydrophobicity. Resolution of these peaks was optimal when less than 2 mg of the material purified on the I-125 column was applied. However, three peaks of interferon activity could be clearly detected when as much as 4 mg of this material was applied to the column. Interferon activity was consistently eluted from this column at a mobile-phase composition between 32 and 41% 1-propanol. When a similar preparation of purified interferon was chromatographed on a Bakerbond C_{18} Wide Pore (300 Å) column under the same solvent conditions, several peaks of interferon activity were eluted from the column (Fig. 3). Three main peaks of activity (fractions 13, 15 and 17) were eluted at a mobile phase composition of between 26 and 33% 1-propanol. No interferon activity was eluted beyond 33% 1-propanol, suggesting that the hydrophobic interactions between interferon and the C_{18} groups of this column are weaker than those between interferon and diphenyl groups on the Protesil column. Resolution of peaks of interferon activity on the Bakerbond C_{18} column was poorer than on the Protesil diphenyl column.

Results of a typical preparation of purified leukocyte interferon are presented in Tables I and II. Crude interferon was purified 1400-fold with a yield of almost 60% by ethanol fractionation and size-exclusion HPLC (Table I). When this material was separated on the Whatman diphenyl column, the pooled peaks of interferon had a specific activity of $8.4 \cdot 10^7$ interferon units per mg protein (Table II, experiment 1)

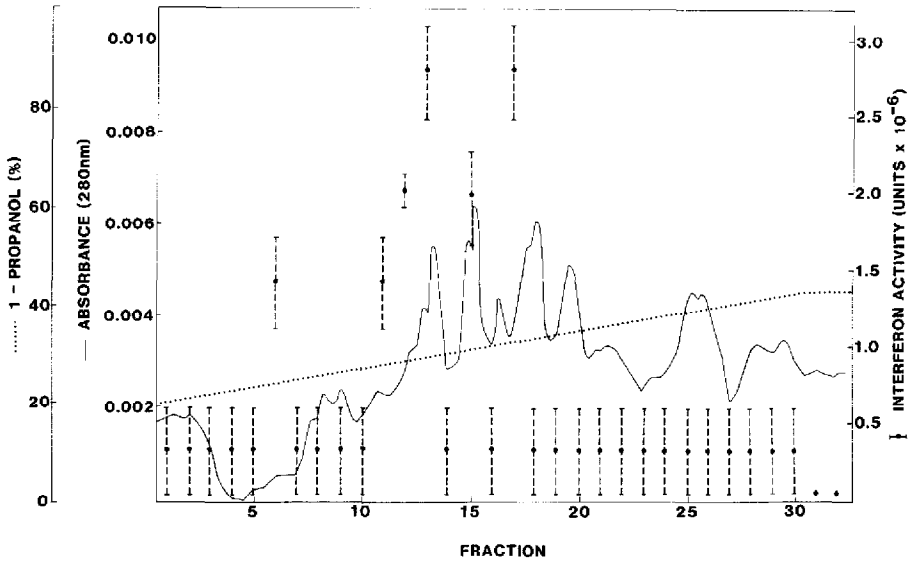

Fig. 3. HPLC separation of leukocyte interferons on a wide-pore C_{18} column. Approximately 0.04 mg of leukocyte interferon purified by ethanol fractionation and size-exclusion HPLC was applied to a Baker-bond Wide Pore C_{18} column that had previously been equilibrated with a mobile phase consisting of 20% Solvent B (1-propanol–2-methoxyethanol, 19:1, pH 2.4) and 80% Solvent A (0.05 *M* KH_2PO_4–2-methoxyethanol, 19:1, pH 2.4). The column was washed for several minutes with the same mobile phase followed by elution of interferon activity with a 45-min gradient from 20 to 45% solvent B in solvent A. The flow-rate was 1.0 ml/min and absorbance was monitored continually at 280 nm with a Waters Model 450 spectrophotometer. Fractions of 1.5 ml were collected and assayed semi-quantitively for interferon activity (inhibition of viral cytopathic effect). Closed circles and broken lines above and below the circles represent the approximate activity for each fraction and the relative error for each determination.

representing a more than 10-fold increase in purity; yield for this step was almost 70%. A slightly greater purification could be achieved by chromatographing the I-125-purified material on a Whatman Protesil 300 C_8 column with a mobile phase containing 24% 1-propanol before applying it to the diphenyl column (Table II, experiment

TABLE I

PREPARATION OF LEUKOCYTE INTERFERONS BY ETHANOL FRACTIONATION AND SIZE-EXCLUSION HPLC

Crude interferon refers to interferon produced as described (see Experimental) and concentrated approximately ten-fold by ultrafiltration prior to ethanol fractionation. The I-125 purified interferon was concentrated by vacuum dialysis against 0.01 *M* phosphate buffer (pH 7.0). Interferon activities were determined by plaque reduction assay as described in the Experimental section.

	Volume (ml)	*Total protein (mg)*	*Total units*	*Specific activity*	*Degree of*	*Yield (%)*
Crude interferon	718	18,668	$1.0 \cdot 10^8$	$5.4 \cdot 10^3$	1	100
Ethanol-fractionated interferon	2.8	66	–	–	–	–
I-125 Purified interferon	8.8	7.6	$5.7 \cdot 10^7$	$7.5 \cdot 10^6$	1400	57

TABLE II

PURIFICATION OF LEUKOCYTE INTERFERON BY REVERSED-PHASE HPLC

The I-125-purified material described in Table I was further purified by diphenyl HPLC as described in the legend to Fig. 2. In Experiment 2, the I-125-purified interferon was chromatographed on a Whatman Protesil C_8 column with a mobile phase consisting of 25% Solvent B (1-propanol–2-methoxythanol, 19:1, pH 2.4) and 75% Solvent A (0.05 *M* KH_2PO_4–2-methoxyethanol, 19:1, pH 2.4) and then concentrated by vacuum dialysis against air prior to diphenyl HPLC.

Experiment	*Step*	*Units applied*	*Units recovered*	*Yield (%)*	*Total protein recovered (mg)*	*Specific activity (units/mg protein)*
1	Diphenyl HPLC	$2.4 \cdot 10^7$	$1.6 \cdot 10^7$	67	0.194	$8.4 \cdot 10^7$
2	C_8/Diphenyl HPLC	$2.1 \cdot 10^7$	$4.0 \cdot 10^7$	100	0.150	$1.4 \cdot 10^8$

2). Apparently interferon is not retained by the column under these conditions while some hydrophobic contaminants are. The final specific activity of the pooled interferons in this case was $1.4 \cdot 10^8$ units per mg protein, representing a 26,000-fold overall increase in purity over the crude interferon.

Samples from each of the purification steps were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (Fig. 4). Diphenyl HPLC-purified interferon was concentrated prior to gel electrophoresis by vacuum dialysis against Dulbecco's phosphate-buffered saline (PBS) buffer. Because this step can result in significant losses of protein, individual peaks of interferon activity containing very small amounts of protein were not analyzed. Instead, all fractions containing interferon activity were pooled, concentrated, and then analyzed by SDS gel electrophoresis. The pooled material contained four major proteins with approximate molecular weights of 14,500, 15,000, 18,500 and 21,000 (lanes 7 and 8). Upon bioassay of fractions cut from the gel, interferon activity was found associated only with the mol.wt. 18,500 and 21,000 proteins. No interferon activity was associated with the mol.wt. 14,500 and 15,000 proteins, although preparations of interferons having molecular weights in this range have been reported[9]. These two proteins were not separated from the mol.wt. 18,500 and 21,000 proteins when the diphenyl HPLC-purified material was chromatographed on a Whatman Protesil C_8 HPLC column (mobile phase as described for diphenyl column; gradient from 14% to 43% 1-propanol).

Rubinstein *et al.*[2,10] have reported the separation of leukocyte interferons into three classes after partial purification by acid precipitation, size-exclusion chromatography on Sephadex G-100, and chromatography on LiChrosorb RP-8 and Diol columns. Further chromatography of the individual interferon classes revealed the presence of several subspecies of leukocyte interferon. It is not known whether the three peaks of activity we have observed after chromatography on the wide-pore diphenyl column contain interferons similar to those reported by Rubinstein. However, the separation reported here appears to be more rapid than that described by Rubinstein; yields in both cases are similar. We are now attempting to analyze individual peaks of interferon activity eluted from the diphenyl column to determine whether unique species of leukocyte interferon may be present.

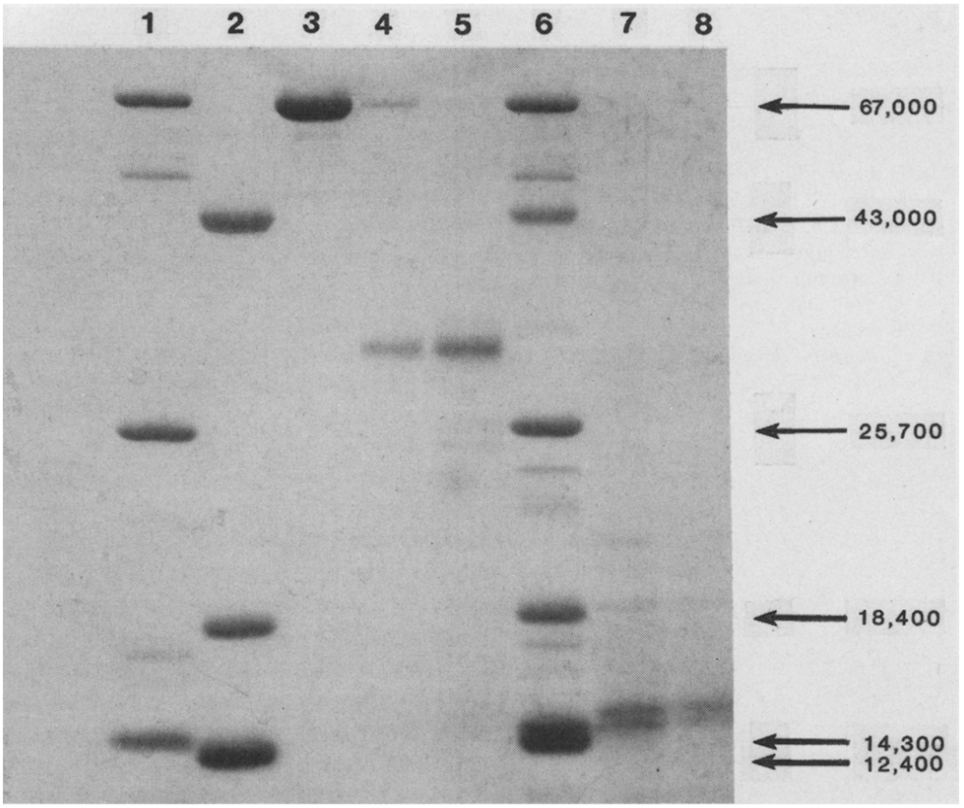

Fig. 4. SDS-Polyacrylamide gel electrophoresis of leukocyte interferon preparations. Preparations of leukocyte interferons at different stages of purity were analyzed by electrophoresis on 12.5% acrylamide gels according to the method of Laemmli[8]. Protein standards and interferon samples were incubated in a solution containing 1% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.13 *M* Tris–HCl (pH 6.8), and 20% (v/v) glycerol for 1 h at room temperature before electrophoresis. After electrophoresis (5 h, 30 mA constant current) one of the lanes (not shown) which contained 7 μg of Diphenyl HPLC-purified interferon was cut off the gel and divided into 2-mm pieces. Each piece was eluted with 0.5 ml of solution containing 0.5 *M* $NaHCO_3$ and 0.1% SDS (37°C, overnight) prior to being assayed for interferon activity (inhibition of viral cytopathic effect). The remaining portion of the acylamide gel slab was stained with a solution containing 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid, and 0.15% (w/v) Coomassie Blue for 2 h and then destained in 5% (v/v) methanol, 10% (v/v) glacial acetic acid. The following amounts of protein were analyzed: lane 1, 6 μg each of the molecular weight markers lysozyme (mol.wt. 14,300), chymotrypsinogen (mol.wt. 25,700), and bovine serum albumin (mol.wt. 67,000); lane 2, 6 μg each of the molecular weight markers cytochrome *c* (mol.wt. 12,400), β-lactoglobulin A (subunit mol.wt. 18,400) and ovalbumin (mol.wt. 43,000); lane 3, 7 μg of crude leukocyte interferon; lane 4, 7 μg of ethanol fractionated leukocyte interferon; lane 5, 7 μg of size-exclusion HPLC-purified interferon; lane 6, 6 μg each of the molecular weight markers cytochrome *c*, lysozyme, β-lactoglobulin A, chymotrypsinogen, ovalbumin, and bovine serum albumin; lane 7, 7 μg of diphenyl HPLC-purified interferon; lane 8, 3.5 μg of diphenyl HPLC-purified interferon.

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