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Purification and partial characterization of human lymphoblastoid interferon

(lower molecular weight component/immunoabsorbance/amino acid composition)

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ABSTRACT **One component of human lymphoblastoid interferon obtained from Namalwa cultures induced by Newcastle disease virus has been purified to a specific activity of 2.5×10^8 interferon units per mg of protein (protein content based on amino acid analysis). A single polypeptide species with an apparent molecular weight of 18,500 comigrating with the antiviral activity was observed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Preliminary amino-terminal sequencing results support the conclusion that the interferon species is essentially homogeneous.**

The purification and characterization of human interferon are currently under investigation because of its clinical potential as an antiviral and antitumor agent (1). Several groups have reported the isolation of homogeneous forms of mouse (2, 3), human leukocyte (4, 5), and human fibroblast interferons (6, 7). Only minute quantities of these apparently homogeneous materials are available for chemical analyses due to the small amounts of interferon produced by induced cells as well as losses incurred during the purification. Thus, only limited data on the amino acid compositions and sequences of interferons have been reported (4, 8, 9). Recent improvements in the large-scale culture of Namalwa cells and the subsequent induction of interferon with Newcastle disease virus, strain B1, have made relatively large quantities of interferon continuously available (10–12). This is a report on the purification of a component of human lymphoblastoid interferon and on its amino acid composition.

MATERIALS AND METHODS

Production of Lymphoblastoid Interferon. The procedures for the culture of Namalwa cells and the induction of interferon have been described (10–12). A portion of the crude interferon used in this study was supplied by Litton Bionetics under National Cancer Institute Contract N01-CO-75380. This material was received as resuspended trichloroacetic acid precipitate as described (11). Newcastle disease virus, strain B1, was prepared by Jack Campbell as reported (10).

Interferon Assay. The antiviral activity of interferon was determined by a modification of the micro method described for rabbit kidney cells (13). This method uses low passage primary culture human foreskin fibroblasts with vesicular stomatitis virus as the challenge. All interferon units are expressed with reference to the National Institutes of Health human interferon standard G-023-901-527.

Preparation of Anti-Interferon Serum. Sheep were immunized with lymphoblastoid interferon in complete Freund's adjuvant over a period of 4 months, following an injection schedule described previously (14). The interferon used was partially purified to a specific activity of $1–5 \times 10^5$ units/mg of protein by immunoabsorbant affinity chromatography using sheep anti-leukocyte interferon coupled to Sepharose 4B. A fraction enriched in gamma globulins was produced from the serum by repeated precipitation with ammonium sulfate at 50% saturation. The gamma globulin fraction was then repeatedly passed over an "impurities column" (fetal calf serum, allantoic fluid with Newcastle disease virus, and homogenized buffy coat bound to Sepharose 4B) to enhance the specificity for anti-interferon (14). The anti-interferon was then coupled to Sepharose 4B (24–36 mg of protein per ml of swollen Sepharose). The typical binding capacity of the anti-interferon was in the range of $4.0–16.0 \times 10^3$ interferon units per mg of coupled protein.

Preparation of Interferon from Uninduced Namalwa Cells. Namalwa cultures were grown and diluted as described (10, 11). The culture supernatant was precipitated with trichloroacetic acid and the purification steps described for induced cultures were followed.

Protein Determination and Amino Acid Analyses. Protein concentrations were determined by the method of Lowry *et al.* (15) with bovine serum albumin as a standard or by amino acid analyses. The analyses were done in the laboratory of P. E. Hare, Geophysical Laboratory, Carnegie Institute of Washington, Washington, DC, using high-performance liquid chromatography and fluorescence detection of *o*-phthalaldehyde amino acid derivatives (16, 17). Proline was measured by gas chromatography as described (18), except that trifluoroacetic anhydride was used rather than pentafluoropropionic anhydride. Cysteic acid and methionine sulfone were measured after performic acid oxidation (19, 20). Tryptophan was determined after hydrolysis in 6 M HCl/4% (wt/vol) thioglycolic acid (21).

PURIFICATION

Concentration of interferon from cell culture medium by precipitation with trichloroacetic acid and the subsequent removal of the trichloroacetate by gel filtration on Sephadex G-25 have been described (11). All columns and collection tubes or vessels used after the Sephadex G-25 column were plastic or siliconized glass.

Affinity Chromatography on Anti-Interferon-Sepharose. The anti-lymphoblastoid interferon columns (275–770 ml) were equilibrated with phosphate-buffered saline, pH 7.4 (P_i/NaCl). The eluant from the previous step (Sephadex G-25), representing 35.2–88.2 g of protein and $0.5–2 \times 10^8$ units of interferon, was applied to each column and washed with 8–24 liters of P_i/NaCl at 6°C. The columns were washed (25°C) with 500–1000 ml of P_i/NaCl and 250–500 ml of McIlvaine's ci-

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; P_i/NaCl, phosphate-buffered saline (4 mM KH_2PO_4/15 mM Na_2HPO_4/133 mM NaCl, pH 7.4) containing 0.05% NaN_3; CBB R-250, Coomassie brilliant blue R-250.

trate/phosphate buffer, pH 5.0 (22). The absorbed interferon was eluted with 500–1000 ml of McIlvaine's citrate/phosphate buffer, pH 2.4–2.6. The interferon was stored at −70°C until enough material was accumulated for the next purification step. For subsequent use the columns were washed first with 250–500 ml of P_i/NaCl, then with 250–300 ml of P_i/NaCl containing 4 M guanidine hydrochloride to remove any remaining bound proteins, and finally with 1–1.5 liters of P_i/NaCl.

Large-Scale Concentration of Affinity-Purified Interferon. The interferon, eluted at pH 2.4–2.6 from the immunoabsorbant affinity columns, was pooled and concentrated on a Pellicon cassette ultrafiltration system (Millipore) PT series, with an ultrafilter having a nominal molecular weight limit of 10,000 and 5 square feet (0.46 m^2) of surface area.

Sephadex G-150. Concentrated interferon (0.5–5.5 × 10^8 interferon units) in 75–100 ml was adjusted to pH 3.5 with 1 M NaOH, applied to a Sephadex G-150 column, and eluted with McIlvaine's citrate/phosphate buffer, pH 3.5, containing 0.05% NaN_3 at 4–6°C.

SP-Sephadex. Operations were carried out at 6°C. All buffers were McIlvaine's citrate/phosphate buffers and contained 0.02% NaN_3.

The pooled Sephadex G-150 fractions containing the antiviral activity were concentrated to 300–450 ml by ultrafiltration, using the Millipore Pellicon cassette system. The concentrate was then applied to a SP-Sephadex column (1.5 cm × 5 cm) that had been equilibrated with pH 3.5 buffer. The column was washed at a flow rate of 1.3 ml/min, first with pH 3.5 buffer and then with pH 4.4 buffer. A subsequent wash with pH 5.5 buffer eluted approximately 90% of the interferon. A final wash with pH 7.0 buffer followed by 0.1 M Na_2HPO_4 buffer at pH 8.0 was required to remove the remaining bound interferon.

Treatment of Interferon with Glycosidase Mixture. Most of the carbohydrate moiety of the interferon was removed by a mixture of glycosidases isolated from a strain of *Streptococcus (Diplococcus) pneumoniae*, type 1, prepared by S. Bose (23). The glycosidase preparation (2.8 mg of protein per ml) contained β-galactosidase (9.4 units/ml), *N*-acetyl-β-galactosaminidase (38 units/ml), and neuraminidase (1.4 units/ml) (24–26). The interferon (pH 5.5) fraction of the preceding SP-Sephadex chromatography was adjusted to pH 6.0 and 5 μl of the glycosidase mixture was added per mg of protein. Incubation proceeded for 4–5 hr at 37°C with shaking.

L-Tryptophyl-L-Tryptophan-Affi-Gel 10 Chromatography. Affi-Gel 10 (lyophilized) was purchased from Bio-Rad. After the treatment of the interferon preparation with the glycosidase mixture described above, the material was adjusted to pH 7.0 with 1 M NaOH and applied to the column at a flow rate of 1.3 ml/min at 6°C. The column (11.3 cm in height; volume, 22.7 ml) was first washed with 100 ml of P_i/NaCl (6°C), and then with 60 ml of P_i/NaCl containing 1 M NaCl (23°C). The absorbed interferon was eluted with 60 ml of P_i/NaCl containing 1 M NaCl and 75% (vol/vol) ethylene glycol (23°C) and collected in an equal volume of 0.1% sodium dodecyl sulfate ($NaDodSO_4$). The interferon eluate was dialyzed against 6-liter volumes of 0.05% $NaDodSO_4$ first for 16 hr (6°C) and then twice for 4 hr (25°C).

Preparation of the Interferon for $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. After elution from the L-tryptophyl-L-tryptophan-Affi-Gel 10 column and dialysis against 0.05% $NaDodSO_4$, the interferon was concentrated to 0.5–1.0 ml by ultrafiltration at 6°C in an Amicon cell equipped with a PM10 membrane. The sample was adjusted to 0.05 M Tris·HCl at pH 6.8, 10% (vol/vol) glycerol, 1% $NaDodSO_4$, and 0.002% bromophenol blue and incubated for 2–3 hr at 25°C prior to electrophoresis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed by using a modification of the Laemmli system (27, 28). The electrophoresis units and sample well forming-combs were purchased from Hoefer Scientific Instruments (San Francisco, CA). Acrylamide and bisacrylamide were purchased from Bio-Rad and $NaDodSO_4$ from Gallard-Schlesinger Chemical Corp. (Carle Place, NY). Preparative gels were 1.5 mm in thickness and 24–27 cm long (five wells). Protein standards of a molecular weight range of 68,000–13,700 [bovine serum albumin (68,000), ovalbumin (43,000), soybean trypsin inhibitor (21,500), myoglobin (16,000), and lysozyme (13,700)] were applied in one well on each slab. Approximately 200–400 μg of protein was applied to each of the remaining wells and the gels were subjected to electrophoresis for 16 hr at 11 mA per slab at 25°C, using a constant temperature bath (Neslab, Portsmouth, NH). The gels were stained for 3–4 hr in 25% (vol/vol) isopropanol/10% (vol/vol) acetic acid/0.025% Coomassie brilliant blue R-250 (CBB R-250), destained with 10% acetic acid with four to five changes over a 3- to 4-hr period, and washed twice with deionized water. The CBB R-250-stained components migrating between the soybean trypsin inhibitor and myoglobin standards were excised and eluted with P_i/NaCl/0.1% $NaDodSO_4$ at 37°C for 16–18 hr. The volume of the elution buffer varied from 2 to 6 ml, depending on the amount of gel to be extracted. The extracted gel was removed by passing each fraction through a Quik Sep column (Isolab Inc., Akron, OH), which was then washed with 1.5 ml of the elution buffer. The eluates were assayed and stored at −70°C. Analytical gels were 0.75 mm thick and 9 cm long.

Table 1. Purification of human lymphoblastoid interferon*

Step	Units recovered × 10^{-6}	Protein, mg	Specific activity, units × 10^{-6}/mg	Recovery range per step, %
Sephadex G-150	128	60.6	2.1	40–60
Ultrafiltration	128	60.6	2.1	90–100
SP-Sephadex C-25†	81	20.1	4.0	65–100
Glycosidase mixture treatment	81	20.4	4.0	100
L-Tryptophyl-L-tryptophan-Affi-Gel 10	72	5.1	14	84–87
$NaDodSO_4$/polyacrylamide gel electrophoresis				
Peak fraction‡	20	0.080	250	27–31
Total	40	ND	ND	55–63

ND, not determined.

* In the experiment described here, total recovery of interferon from trichloroacetic acid precipitate (7.8 × 10^8 units) to the peak fraction of the gel is approximately 3%. Modifications in storage and handling procedures described in the *Results* have increased the overall recovery to approximately 10%.

† pH 5.5 eluate only.

‡ The amount of interferon, estimated by amino acid analysis, recovered in the polypeptide species with the apparent molecular weight of 18,500.

RESULTS

A human lymphoblastoid interferon with an apparent molecular weight of 18,500 has been purified to a single component by $NaDodSO_4$/polyacrylamide gel electrophoresis and has a specific activity of 2.5×10^8 units/mg of protein. Its production, concentration by precipitation, and gel filtration on Sephadex G-25 have been described (11). The subsequent immunoabsorbant-affinity chromatography step was modified by substituting anti-lymphoblastoid interferon for anti-leukocyte interferon (11). The purification using either antibody was essentially the same. Three to 10% of the antiviral activity did not bind to the column. An average of 70% was eluted with McIlvaine's citrate/phosphate buffer, pH 2.4–2.6, and 20% or less was eluted with 4 M guanidine hydrochloride in P_i/NaCl. The interferon eluted at pH 2.4–2.6 had a specific activity of $1–8 \times 10^5$ units/mg of protein (100- to 800-fold purification over the trichloroacetic acid precipitate) and was antigenically the leukocyte subspecies. It was stored at −70°C until $2–5 \times 10^8$ units were accumulated. The interferon was then thawed, pooled, and concentrated by ultrafiltration using the Pellicon cassette system. The concentrate was fractionated by gel filtration on Sephadex G-150. An elution profile is shown in Fig. 1. Less than 5% of the antiviral activity was associated with proteins eluting at the void volume. Approximately 40–60% of the antiviral activity was recovered in the volume eluted between 3800 and 5000 ml. This pooled eluant had a protein concentration of 23–27 μg/ml.

Interferon eluted from the Sephadex G-150 column was concentrated by ultrafiltration with no loss of antiviral activity. A summary of the purification is shown in Table 1. We have experienced losses of antiviral activity up to 60% in partially purified interferon preparations when protein at concentrations below 100 μg/ml was stored at 6°C or −70°C in plastic bottles or tubes. Collection of interferon fractions with these low protein concentrations in siliconized glass bottles followed by immediate concentration using ultrafiltration (Pellicon cassette system previously described) has virtually eliminated these losses. Thus, the overall recovery of the interferon species with a molecular weight of 18,500 is 5–10% rather than the 3% reported in Table 1 for a batch that had incurred these adsorption losses.

After gel filtration on Sephadex G-150 the material was further purified by ion-exchange chromatography using SP-Sephadex C-25. Essentially 100% of the interferon activity was bound to the SP-Sephadex column at pH 3.5. Less than 1% of the interferon was eluted at pH 4.4. Approximately 90% of the recovered interferon was eluted at pH 5.5 and 10% at pH 7.0. Fig. 2, lanes 1 and 2, shows the CBB R-250-stained polypeptide patterns obtained with interferon-containing fractions eluted from Sephadex G-150 and SP-Sephadex columns, respectively. Analytical $NaDodSO_4$/polyacrylamide gels of the SP-Sephadex fractions showed that several polypeptide species migrating in the region of the antiviral activity were removed by the ion-exchange chromatography (results not shown). Treatment of the interferon preparation with the glycosidase mixture resulted in complete recovery of the antiviral activity. No component of the glycosidase preparation appeared to comigrate with the interferon polypeptide species when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

The dipeptide L-tryptophyl-L-tryptophan, bound to a solid-phase support, has been used in the purification of human leukocyte interferon (29). This chromatography was effective in purifying lymphoblastoid interferon. However, the high

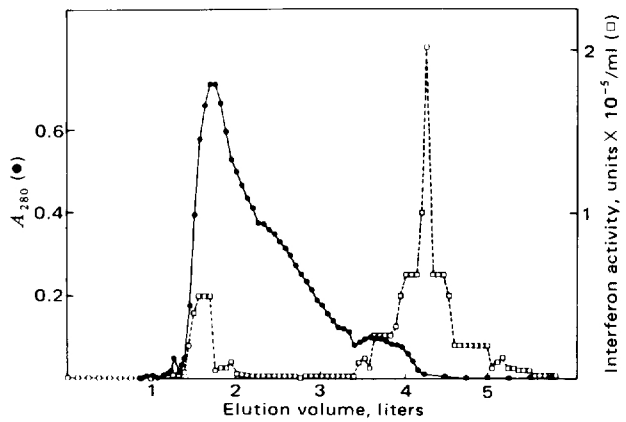

FIG. 1. Purification of lymphoblastoid interferon by Sephadex G-150. The immunoabsorbant affinity eluates (3×10^8 units, 867 mg of protein) were concentrated, adjusted to pH 3.5, and applied to the column (46.5 cm in height, 6200 ml in volume). Fractions (12–13 ml) were collected at a flow rate of 60 ml/hr at 4–6°C. The peak fractions of interferon contained 1.5×10^8 units and 52.3 mg of protein.

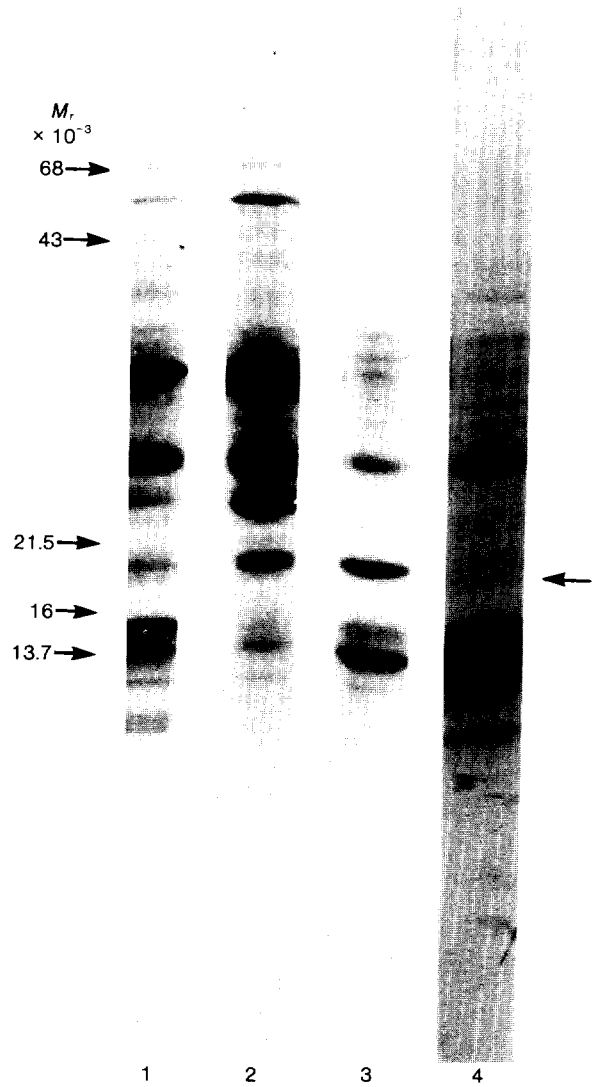

FIG. 2. Analytical $NaDodSO_4$/polyacrylamide gel electrophoresis of interferon from induced and uninduced cultures after several steps in the purification. Lanes 1–3 show the interferon from Newcastle disease virus-induced cells and lane 4, interferon from uninduced cells. Marker proteins designated by arrows on left are described in *Materials and Methods*. The arrow on the right indicates the component corresponding to the antiviral activity. Lane 1, Sephadex G-150 fraction; lane 2, SP-Sephadex fraction, pH 5.5; lanes 3 and 4, ethylene glycol fractions of L-tryptophyl-L-tryptophan-Affi-Gel 10.

concentration of ethylene glycol necessary to elute the interferon also appeared to cause a loss of antiviral activity. Therefore, the interferon eluted with 75% ethylene glycol was collected in an equal volume of 0.1% $NaDodSO_4$. The reduction of the ethylene glycol concentration and the addition of $NaDodSO_4$ stabilized the interferon. The stabilization of interferon with low concentrations of $NaDodSO_4$ was also observed with fibroblast interferon and leukocyte interferon (6, 30, 31). Fig. 2, lane 3, shows an analytical $NaDodSO_4$ gel of the interferon preparation after L-tryptophyl-L-tryptophan-Affi-Gel 10 chromatography.

In order to support that the polypeptide with the molecular weight 18,500 associated with the antiviral activity was in fact related to induction, interferon was prepared from uninduced cultures, which had been shown to produce low levels of interferon constitutively (32). The supernatant of 200 liters of uninduced Namalwa cells contained 2.5×10^5 units of interferon, which represents 0.25% of the amount obtained with a virus-induced culture. The specific activity of the interferon from the uninduced culture after gel filtration on Sephadex G-25 was 4 units/mg of protein. The purification of this interferon was analogous to that of the interferon from induced cultures. Fig. 2, lane 4, shows the CBB R-250-stained pattern of the ethylene glycol eluate of the L-tryptophyl-L-tryptophan column. The intensity of the stained component with a molecular weight of 18,500 from the uninduced culture was greatly diminished. This correlates with the difference in the specific activities of the two ethylene glycol fractions, the partially purified interferon from uninduced cells having a specific activity of 2.5×10^5 units/mg of protein.

Fig. 3 shows a typical preparative $NaDodSO_4$ gel, and the comigration of the 18,500 component with antiviral activity. The gel fractions corresponding to this active polypeptide species were excised, eluted, and analyzed by analytical $NaDodSO_4$/polyacrylamide gel electrophoresis as shown in Fig. 4, lane 2. The amino acid composition of the polypeptide is shown in Table 2. The analyses were performed with 1- to 3-μg samples, which were analyzed directly or dialyzed twice against 6-liter volumes of P_i/NaCl containing 0.1% $NaDodSO_4$ and then twice against 1-liter volumes of 0.01% $NaDodSO_4$ prior to analysis. Unhydrolyzed samples were analyzed to monitor for possible contamination and the quantity of each amino acid detected was less than 10% of that found in the hydrolyzed samples. These levels were subtracted from the values observed with the hydrolyzed samples. The value obtained for methionine sulfone is a minimum value because of losses in oxidation (20). Sequencing data on the amino-terminal region of this component obtained in collaboration with M. Hunkapiller and L. Hood of the California Institute of Technology, Pasadena, CA, indicate an essentially homogeneous polypeptide (to be published). A second polypeptide representing less than 10% of the total amount of protein was detected during the first few steps of sequencing of one preparation; however, with a second preparation, a single sequence was obtained. The quantity of each amino acid phenylthiohydantoin derivative released correlated with the amount of protein analyzed, strongly indicating that this component of lymphoblastoid interferon is not blocked at its amino terminus and is the protein being sequenced.

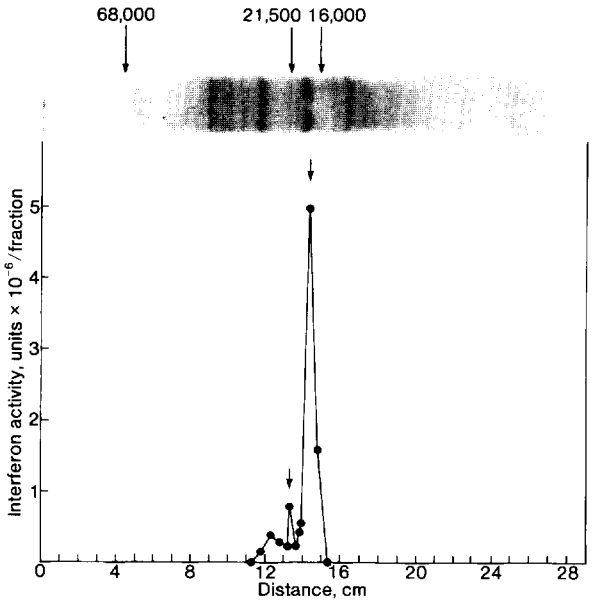

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of partially purified interferon. Interferon (1.9×10^7 units, 1.2 mg of protein) was applied to the gel. Total recovery of interferon was 9.7×10^6 units with 5.0×10^6 units in the 18,500 species. The CBB R-250 polypeptide pattern on the top of the figure represents one lane of preparative gel.

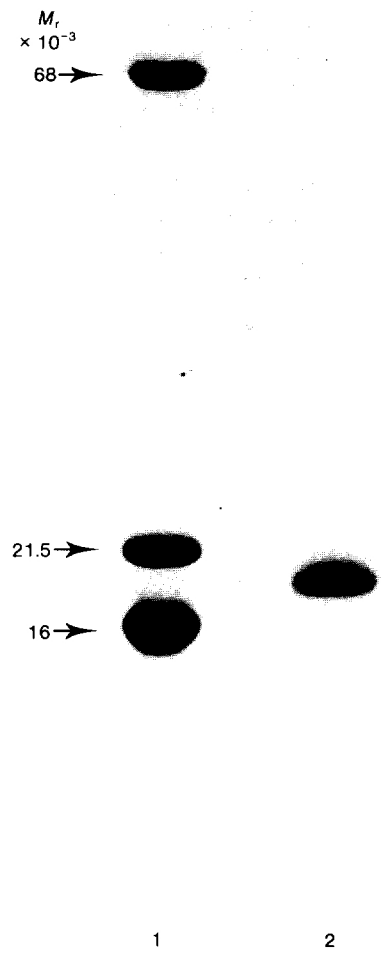

FIG. 4. Analytical $NaDodSO_4$/polyacrylamide gel electrophoresis of the 18,500 species of lymphoblastoid interferon. Lane 1, molecular weight standards: 2.5 μg of bovine serum albumin (68,000) and 5.0 μg each of soybean trypsin inhibitor (21,500) and myoglobin (16,000). Lane 2, 2.2 μg of 18,500 species derived from peak fraction of $NaDodSO_4$ preparative gel (see Table 1 and Fig. 3).

Table 2. Amino acid composition of human lymphoblastoid interferon

Amino acid residue	Residues per 155 amino acid residues*
Asx	14.0
Thr	7.5
Ser	10.0
Glx	25.6
Pro†	10.2
Gly	10.0
Ala	10.3
Cys‡	1.7
Val	7.2
Met§	1.1
Ile	6.5
Leu	16.7
Tyr	3.6
Phe	6.7
His	4.1
Lys	9.8
Arg	9.0
Trp	0.6

* On the basis of a molecular weight of 18,500, the number of amino acid residues is 155. The number of residues of each amino acid represents an average of four determinations, except for Pro, Cys, Met, and Trp.

† Measured by gas chromatography; two determinations.

‡ Measured after performic acid oxidation as cysteic acid; two determinations.

§ Measured after performic acid oxidation as methionine sulfone; one determination.

DISCUSSION

A single component of human lymphoblastoid interferon has been purified over 100,000-fold to homogeneity. The success of its purification can be attributed to recent developments in the large-scale production of lymphoblastoid interferon (11, 12). The preparation of large quantities of crude interferon has made it possible to purify sufficient quantities of homogeneous 18,500 polypeptide to permit the initial characterization studies.

The purified interferon is enriched in hydrophobic amino acids and is very similar in composition to the mouse and human fibroblast and leukocyte interferons (4, 8, 9). All these interferons are characterized by large quantities of leucine, aspartic acid/asparagine, and glutamic acid/glutamine. Attempts to identify the amino terminus of human lymphoblastoid interferon by dansylation were unsuccessful primarily because of contaminating amino acids present in the purified interferon preparation (33), and initial sequence studies had shown multiple phenylthiohydantoin derivatives in the first two cycles. Subsequent cycles had yielded essentially one sequence. In recent interferon preparations, the contaminants were removed and a single sequence for the amino-terminal region was obtained.

Sequencing studies on the purified interferon, using both liquid and solid-phase sequencers, are not yet complete. A comparison of the biological properties as well as the amino acid sequences of the purified human lymphoblastoid, leukocyte, and fibroblast interferons, and mouse interferons should soon be possible. In addition, it is hoped that the imminent availability of large amounts of purified lymphoblastoid interferon will permit more extensive examination of the antitumor, antiviral, immunosuppressive, and growth inhibition effects reported for partially purified interferon.

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