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Purification of Human γ-Interferon to Essential Homogeneity and Its Biochemical Characterization†

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ABSTRACT: A multistep procedure has been developed which enables human γ-interferon (HuIFN-γ) to be purified to essential homogeneity. The procedure takes advantage of a modification of a previously described sequential chromatographic technique [Braude, I. A. (1983) *Prep. Biochem. 13*, 177–190] and the high isoelectric point of HuIFN-γ (pH 9.5–9.8). The steps include Controlled Pore Glass adsorption chromatography, concanavalin A–Sepharose and heparin–Sepharose affinity chromatography, cation-exchange chromatography, and gel filtration chromatography. The purified HuIFN-γ had a specific activity of 5.9×10^7 units/mg. This represents a purification of more than 70 000-fold and a 33% recovery. In addition, one gel filtration fraction had a specific activity of 2.5×10^8 units/mg. This represents a purification of greater than 300 000-fold and a recovery of greater than 17%. This fraction, when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, was shown to be composed of one major 26-kilodalton (kDa) species and four minor species of 74, 67, 56, and 22 kDa. Analysis of this material with anti-HuIFN-γ monoclonal antibody immunoabsorbent columns indicates that both the 26- and the 22-kDa species are HuIFN-γ. Thus, the final product is essentially homogeneous (90–92% HuIFN-γ), and the specific activity of pure HuIFN-γ is approximately $(2.7–2.8) \times 10^8$ units/mg of protein. Finally, the 26- and 22-kDa moieties are shown to be similar, if not identical, proteins as judged by amino acid and sequence analyses.

Interferons (IFN's)[1] are a family of proteins which possess a variety of biological properties. Most notable are its antiviral (Isaacs & Lindenmann, 1957), antiproliferative (Gresser et al., 1970; Strander & Einhorn, 1977), and immunoregulatory (Johnson, 1978; Herberman et al., 1979; Hernandez-Asensio et al., 1979; Minato et al., 1980) characteristics. The most recent interferon class to be discovered is IFN-γ (Wheelock, 1965; Falcoff et al., 1972; Salvin et al., 1973; Epstein, 1976).

Unlike IFN-α and IFN-β, IFN-γ is primarily produced by antigen- or mitogen-stimulated T lymphocytes. IFN-γ has also been shown to be physiochemically (Langford et al., 1979; Yip et al., 1981; Gray et al., 1982) and antigenically (deLey et al., 1980; Wiranowski-Stewart et al., 1980) distinct from IFN-α and IFN-β. However, similar to IFN-α and IFN-β, IFN-γ induces or alters many of the same intracellular metabolic events such as the (2′–5′)-oligoadenylate synthetase (Baglioni & Maroney, 1980) and protein kinase systems (Falcoff et al., 1980), as well as a variety of de novo synthesized proteins (Rubin et al., 1980).

Interest in the IFN-γ system has increased since data describing its potent antiproliferative (deLey et al., 1980; Rubin & Gupta, 1980), immunoregulatory (Basham & Merigan, 1983), and (when used in combination with either IFN-α or IFN-β) potentiating (Fleischmann et al., 1979; De Clercq et al., 1982) properties were reported. In addition, clinical studies to evaluate HuIFN-γ's antitumor properties are being conducted (Gutterman et al., 1983; Oldham et al., 1983).

Further studies regarding the biological and biochemical properties of HuIFN-γ require highly purified preparations. In this report, a procedure is described for the purification of HuIFN-γ to essential homogeneity. Furthermore, employing a variety of immunochemical and biochemical techniques, it is shown that HuIFN-γ is found as at least two molecular weight forms whose polypeptide structures are similar if not identical.

Materials and Methods

Resins. Controlled Pore Glass beads (350-Å pore size, 120–200 mesh) were purchased from Electro-Nucleonics (Fairfield, NJ). Concanavalin A–Sepharose, heparin–Sepharose, and cyanogen bromide activated Sepharose were supplied by Pharmacia Fine Chemicals (Piscataway, NJ). Carboxymethyl-Bio-Gel-agarose was obtained from Bio-Rad (Richmond, CA), while the Ultrogel AcA 54 was obtained form LKB (Rockville, MD).

Interferon and Anti-interferon Monoclonal Antibodies. HuIFN-γ was produced as previously described (Braude, 1983a). Briefly, leukocytes from human buffy coats were induced with a combination of A-23187 (0.5 μg/mL) and

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[1] Abbreviations: IFN, interferon; HuIFN-α, human α-interferon; HuIFN-β, human β-interferon; HuIFN-γ, human γ-interferon; CPG, Controlled Pore Glass; Con A/S, concanavalin A–Sepharose; H/S, heparin–Sepharose; PBS, phosphate-buffered saline; PB, 20 mM phosphate buffer, pH 7.2; CM-BGA, carboxymethyl-Bio-Gel–agarose; α-MM, methyl α-D-mannopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton(s); IEP, isoelectric point; Tris, tris(hydroxymethyl)aminomethane; pfu, plaque-forming unit(s); TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

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mezerein (70 ng/mL). After incubation at 37 °C in 5% CO_2 for 72 h, the crude material was harvested and clarified by centrifugation at 2600*g* for 1 h and stored at 4 °C. The material was brought to pH 9.5 with 0.5 M Tris and reclarified at 2600*g* for 1 h.

Two monoclonal antibodies (C-57 and C-22) were produced by the methods previously described by Kohler & Milstein (1975). Both monoclonal antibodies are of the IgG_1 subclass and neutralize the antiviral properties of HuIFN-γ (R. Gillette and I. Braude, unpublished results).

Interferon Assay. The interferon end point was determined by a cytopathic effect inhibition assay (Tilles & Finland, 1968; Armstrong, 1971). Samples were assayed in 96-well microtitration plates (Falcon, Oxnard, CA). Serial 3-fold dilutions of samples were made in Dulbelco's minimal essential medium containing 10% fetal calf serum (Meloy Laboratories, Springfield, VA). Trays were then sterilized by UV irradiation. WISH cells (2.5×10^4 cells/well; American Type Culture Collection, Rockville, MD) were added, and the trays were incubated overnight at 37 °C in 5% CO_2. Cells were then challenged with encephalomyocarditis virus ($\sim 2.0 \times 10^3$ pfu/well) and incubated at 37 °C in 5% CO_2 for 18 h.

All interferon titers were expressed in terms of international units per milliliter. This was accomplished by calibrating the international standard (G-g23-901-530) against the Meloy internal standard. Twenty-seven assays of both standards were performed in parallel. The geometric mean titer for the international standard was 3563 units/mL (range 2742–4628 units/mL). The geometric mean titer for the internal standard was 3988 units/mL (range 2922–5445 units/mL).

Protein Determinations. All protein determinations were made by utilizing the Bio-Rad (Richmond, CA) Coomassie micro dye-binding assay, as originally described by Bradford (1976).

Chromatographic Procedures. The chromatographic purification of crude HuIFN-γ was a modification of a previously described technique (Braude, 1983b). Crude HuIFN-γ (adjusted to pH 9.5) was loaded onto a 5.0 × 20.4 cm column of CPG, at a flow rate of 900 mL/h. The column was then sequentially washed (at 900 mL/h) with 0.5 M Tris-HCl, pH 9.5, and PBS until the absorbance at 280 nm (A_{280}) of each reached the base line. Finally, the HuIFN-γ was eluted, at a flow rate of 200 mL/h, with 2 M $(NH_4)_2SO_4$, pH 9.0.

The second protein peak, eluted from the CPG with $(NH_4)_2SO_4$, pH 9.0, was loaded at a flow rate of 200 mL/h directly onto a 2.6 × 18.9 cm column of Con A/S. After the column was loaded (i.e., the A_{280} returned to base the line), the Con A/S column (with the CPG column disconnected) was washed with an additional 3 column volumes of 2 M $(NH_4)_2SO_4$, pH 9.0. The resin was then washed, at a flow rate of 20 mL/h, with PBS until the A_{280} returned to the base line. Finally, the HuIFN-γ was eluted, at a flow rate of 64 mL/h, with PBS containing 1.0 M α-MM.

The Con A/S eluted HuIFN-γ was loaded, at a flow rate of 64 mL/h, directly onto a 2.6 × 7.5 cm column of H/S. After the HuIFN-γ was loaded (i.e., A_{280} returned to the base line) and the Con A/S column was disconnected, the H/S was washed with 1 column volume each of PB and PBS. The HuIFN-γ was eluted, in an ascending direction, with PBS containing 2 M NaCl. Three-milliliter fractions were collected, and the peak fractions were pooled and dialyzed against 10 mM Tris-HCl, pH 9.5.

The dialyzed H/S-purified material was loaded, at a flow rate of 20 mL/h, onto a 1.6 × 10.0 cm column of CM-BGA equilibrated in 10 mM Tris-HCl, pH 9.5. The resin was washed in the same buffer until the A_{280} returned to the base line. The HuIFN-γ was eluted, in an ascending direction, with 20 mM Tris-HCl, pH 9.5, containing 50 mM NaCl. Two-milliliter fractions were collected, and fractions spanning the two eluted protein peaks were pooled. Finally, the column was washed with 20 mM Tris-HCl, pH 9.5, containing 2 M NaCl, and 2-mL fractions were collected.

The pooled fractions were loaded, at a flow rate of 20 mL/h onto a pair of 2.6 × 90 cm columns of Ultrogel AcA 54 equilibrated in PBS containing 2 M NaCl. Approximately 10-mL fractions were collected.

SDS–PAGE. Samples were analyzed by SDS–PAGE as previously described by Laemmli (1970). Briefly, samples were prepared in 10 mM Tris-HCl, pH 6.8, with 0.1% (v/v) SDS, brought to a final concentration of 1.0% (v/v) SDS, and heated at 95 °C for 1 min. After being cooled, samples were loaded onto a 28 cm long × 17.5 cm wide × 0.15 cm thick slab gel. The stacking gel was 1 cm long and consisted of 3.1% acrylamide, and the separating gel was approximately 18 cm long and composed of 12.0% acrylamide. The samples were electrophoresed at 15 mA into the separating gel, at 20 mA for 1 cm into the separating gel, and at 25 mA until the bromophenol blue dye front had migrated at least 10 cm.

Stained gels were treated with 0.25% Coomassie blue R-250 (Bio-Rad, Richmond, CA; 1:1:2.5:5.5 Coomassie:acetic acid:2-propanol:water) overnight and destained (1:9 acetic acid:water) for 24 h. After the gels were destained, they were scanned at 565 nm in a Beckman DN-8 spectrophotometer (Palo Alto, CA).

Autoradiograms of the appropriate gels were performed at −70 °C with Kodak XA-5 film (Eastman Kodak, Rochester, NY) in combination with a Cronex intensifier (Du Pont, Wilmington, DE).

Preparative gels were performed in 1.46 cm in diameter × 12.5 cm long cylindrical tubes. The stacking gel was 1 cm long and composed of 3.1% acrylamide, and the separating gel was 11 cm long and composed of 12% acrylamide. Approximately 500–1000 μL of sample (prepared as described above) was electrophoresed at 20 mA/tube until the bromophenol blue dye front had migrated at least 10 cm. The gels were extruded (LAC Enterprises, Santa Barbara, CA) and stained.

The molecular weight markers (LMW calibration kit) were from Pharmacia (Piscataway, NJ).

Isoelectric Focusing. The isoelectric focusing of HuIFN-γ was a modification of the technique previously described by O'Farrell (1975). Briefly, gel tubes 2.5 mm in diameter × 14 cm long contained 9 M urea, 3.8% acrylamide, 0.2% bis-(acrylamide), and 2% (v/v) ampholine (LKB, Rockville, MD; 2:1:1:1:3 mixtures of pH 5–8, 6–8, 7–9, 8–9.5, and 9–11 ampholines, respectively). The gels were polymerized by the addition of TEMED (7 μL/10 mL of H_2O) and ammonium persulfate (1 μ g/10 μL of H_2O). The interferon samples were prepared in focusing sample buffer containing 9.5 M urea, 5% 2-mercaptoethanol, and 2% ampholines (see above). Approximately 40 μL of sample was applied to a prerun gel (200 V for 0.5 h, 200 V for 0.5 h, and 400 V for 0.5 h). The samples were then run at 400 V for 8 h. Finally the gels were extruded, and 2-mm segments were incubated at 4 °C overnight in assay medium (see above) and assayed for antiviral activity. One-centimeter segments of a companion gel were incubated overnight in distilled water, and the pH was measured.

Immunoabsorbents. Mouse monoclonal antibodies (derived from ascites fluid) were partially purified by repeated 40% saturation $(NH_4)_2SO_4$ precipitations. This material was then

immobilized onto cyanogen bromide activated Sepharose 4B as previously described by Pharmacia (1974). A control column containing partially purified [by $(NH_4)_2SO_4$ precipitations] monoclonal antibodies directed against theophylline was prepared in a similar fashion. Both resins contained approximately 2.5 mg of protein/mL of gel.

Approximately 5.0×10^6 units (20 μg) of highly purified HuIFN-γ was radiolabeled with Iodogen (Pierce Chemical, Rockford, IL) as previously described (Crowther & Harness, 1983). This material was exhaustively dialyzed against PBS and applied to either 1 mL of anti-HuIFN-γ (I-57 and I-22) or anti-theophylline (I-T) monoclonal antibody of immunoabsorbent columns. After the columns were loaded, they were washed with 10 mL of PBS. Finally, the columns were washed with PBS containing 1.0% SDS. One-milliliter fractions were collected and counted in a Packard 5260 γ counter (Downers Grove, IL), and the peak fractions were analyzed by autoradiography of SDS–PAGE (see above).

Amino Acid Analysis. Stained bands were excised from preparative SDS–PAGE gels (see above) and pulverized in a tissue grinder containing 10 mL of 10 mM Tris-HCl, pH 9.5, 0.1% SDS, and 0.1% sodium azide. The material was transferred to a 50-mL Falcon 2070 conical centrifuge tube (Oxnard, CA) and vigorously agitated, at 37 °C, on an orbital shaker for 18 h. The soluble material was separated from the gel by passage through QS-1 Quik-Sep (Isolab, Akron, OH) disposable columns, exhaustively dialyzed against H_2O, and freeze-dried.

Samples for analyses were hydrolyzed, for 18 h, in 6 N HCl, at 110 °C, under vacuum. The hydrolysates were evaported to dryness and taken up in 0.2 M sodium citrate buffer, pH 2.2. Appropriate aliquots were analyzed in a Dionex (Durrum) D-500 amino acid analyzer (Sunnyvale, CA).

Amino Acid Sequence. Stained gel bands (from preparative gels) were prepared as described above. The material was then reconstituted in 70% formic acid and digested, while being stirred in an amber-colored Reacti-Vial (Pierce Chemicals, Rockford, IL) for 72 h, with a 150 molar excess of cyanogen bromide (Kodak Chemicals, Rochester, NY). The reaction was terminated by blowing off the formic acid with air, adding H_2O, and freeze-drying.

Sequence analyses were performed on both a Beckman 890C spinning-cup sequencer (Palo Alto, CA) and an Applied Biosystems Model 470A gas-phase sequencer (Foster City, CA). The spinning-cup sequencer program was adapted from the work of Brauer et al. (1975), and the gas-phase sequencer program was essentially as described by Hunkapiller & Hood (1978). The phenylthiohydantoin derivatives were analyzed on a Hewlett-Packard 1084B high-performance liquid chromatographic system (Palo Alto, CA).

Results

Isoelectric Focusing of HuIFN-γ. In a previous report (Braude, 1983b), it was shown that HuIFN-γ could be purified on the basis of glass absorption, sugar–lectin interactions, binding to polysulfonated polymers, and size. In consideration of the purification of HuIFN-γ by charge interactions, the isoelectric point of HuIFN-γ was first determined by isoelectric focusing.

As shown in Figure 1, the IEP of partially purified HuIFN-γ was between 9.5 and 9.8. This value represented 6% of the applied bioactivity. The use of different pH gradients did not significantly alter this value (data not shown).

Purification of HuIFN-γ. Improvements in the purification of HuIFN-γ were made by taking advantage of HuIFN-γ's high IEP (see Figure 1) and modifying the H/S buffers. The addition of a cationic exchange column to the modified purification procedure yielded essentially homogeneous material.

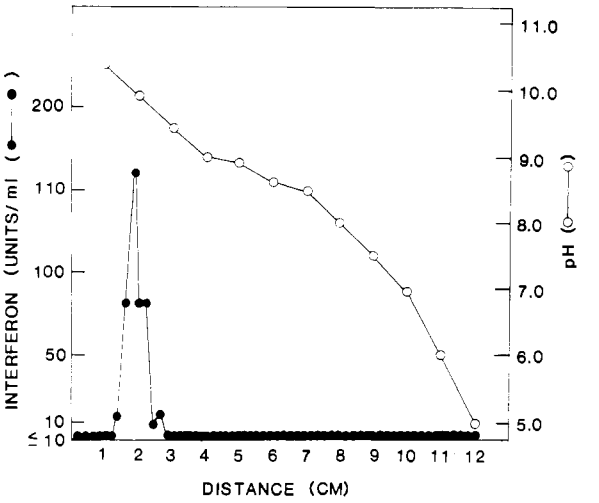

FIGURE 1: Isoelectric point of HuIFN-γ. Approximately 6000 units (in 40 μL) of HuIFN-γ was loaded onto a cylindrical isoelectric focusing gel. After the run, the gel segments were incubated, and either they were assayed for bioactivity (●) or the pH was measured (○).

An example of this modified procedure is shown in Figure 2. Panel A represents the sequential chromatographic portion of the procedure. Due to its sequential nature, the amount of HuIFN-γ eluted onto the subsequent columns was not determined, and therefore, only the breakthrough and the final H/S elution activities are indicated.

As previously shown (Braude, 1983b), the first step in the procedure was CPG absorption chromatography (section I in panel A of Figure 2). Sixteen liters of crude material (previously treated with Tris and adjusted to pH 9.5) was loaded onto the column. Approximately 94% of the bioactivity was retained while the bulk of the contaminants passed unbound. Additional proteins, but not HuIFN-γ, eluted from the column after being washed with PBS. Finally, the material was eluted with 2 M $(NH_4)_2SO_4$, pH 9.0, and 11% of the bioactivity was detected in the first peak and the balance, which was loaded directly onto the Con A/S (section II) column, in the second peak.

Only trace amounts of bioactivity (0.5%) and protein were observed to pass unbound through the Con A/S column. Furthermore, upon washing with PBS, the majority of the protein eluted while only 7% of the bioactivity could be detected. Finally, the bulk of the activity and some additional proteins eluted from the column with 1.0 M α-MM. This material was loaded directly onto the H/S column (section III).

Of the material loaded on the H/S column, only marginal levels of proteins and bioactivity (0.02%) were detected in the breakthrough fractions. The column was then washed with PB and PBS. These two buffers eluted additional protein but only 0.4% and 0.0% (i.e., no activity detectable) of the bioactivity, respectively. The remaining HuIFN-γ and proteins were then eluted with 2 M NaCl.

As shown in Table I, of the 3.2×10^8 units and 384000 mg of protein (specific activity 8.3×10^2 units/mg) applied to this sequential part of the purification procedure, a total of 3.0×10^8 units and 57.6 mg of protein (specific activity 5.2×10^6 units/mg) were recovered. This represented a recovery of 94% and a purification of greater than 6000-fold. The predicted recovery, by summing the breakthrough fractions, was 76%. Given the variation in the bioassay, this value is in close agreement with the percent recovery stated above.

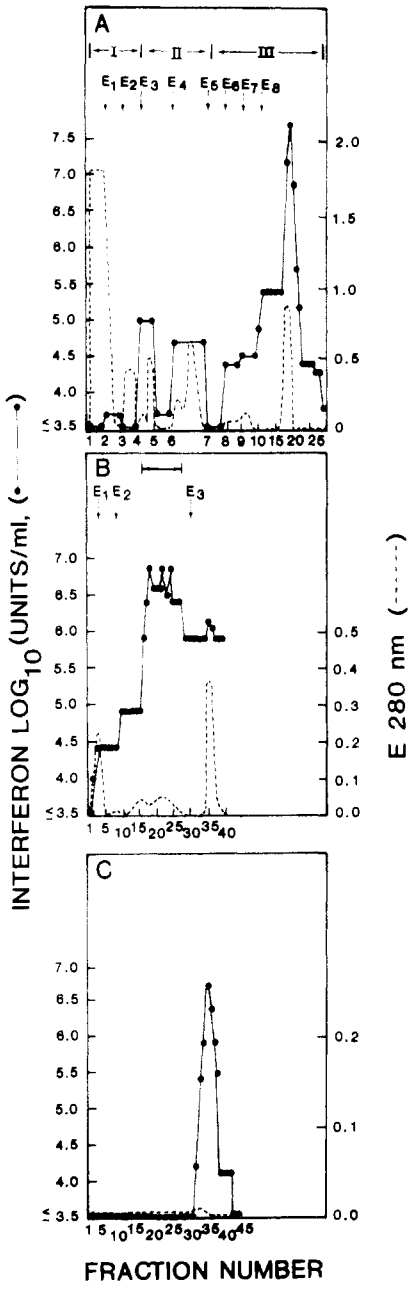

FIGURE 2: Chromatographic purification of HuIFN-γ. Crude HuIFN-γ was initially purified by a three-step sequential chromatographic procedure (A). The first step (section I) was CPG absorption chromatography. The second step (section II) was Con A affinity chromatography. The third step (section III) was H/S affinity chromatography. All fractions were assayed for bioactivity (●), and the protein content was continuously monitored at 280 nm (---). The eluants were (E_1) 0.5 M Tris-HCl, pH 9.5, (E_2) PBS, (E_3) 2 M $(NH_4)_2SO_4$, pH 9.0, (E_4) PBS, (E_5) PBS containing 1.0 M α-MM, (E_6) PB, (E_7) PBS, and (E_8) PBS containing 2 M NaCl. The second part of the purification procedure was cationic exchange chromatography (B). After exhaustive dialysis, the H/S-purified material was further purified on CM-BGA. All fractions were assayed for bioactivity, and the protein was monitored as described above. The eluants were (E_1) 10 mM Tris-HCl, pH 9.5, (E_2) 20 mM Tris-HCl, pH 9.5, containing 50 mM NaCl, and (E_3) 20 mM Tris-HCl, pH 9.5, containing 2 M NaCl. The arrow bordered by two vertical lines represents the fractions pooled and loaded onto the gel filtration column. The last part of the procedure was gel filtration chromatography (C). High-titered CM-BGA fractions were pooled and loaded directly onto two Ultrogel AcA 54 columns hooked in series. All fractions were assayed for bioactivity, and the protein was monitored as described above.

The second part of the procedure (cation-exchange chromatography) is shown in Figure 2B. Approximately 30% of the applied protein and 0.2% of the bioactivity were detected in the breakthrough fractions. The majority of the recoverable HuIFN-γ and approximately 6% of the protein were obtained after the column was washed with 20 mM Tris-HCl, pH 9.5, containing 50 mM NaCl. The balance of the recoverable activity (approximately 8%) and protein were obtained when the column was washed with 20 mM Tris-HCl, pH 9.5, containing 2 M NaCl.

As indicated in Table I, of the 3.0×10^8 units and 47.6 mg of protein (specific activity 5.2×10^6 units/mg) applied to the CM-BGA, a total of 9.8×10^7 units and 3.4 mg of protein (specific activity 2.9×10^7 units/mg) were obtained from the 20 mM Tris-HCl, pH 9.5, containing 50 mM NaCl wash and were usable in the last step of the purification procedure. This represented a recovery of 31% and an overall purification of greater than 34 000-fold.

The last step of the procedure (gel filtration) is shown in Figure 2C. Unlike the previous report (Braude, 1983b), the bioactivity and protein coeluted. In addition, no other major protein peaks were observed.

The results in Table I show that of the 9.8×10^7 units and 3.4 mg of protein (specific activity 2.9×10^7 units/mg) a total of 1.0×10^8 units and 1.69 mg of protein (specific activity 5.9×10^7 units/mg) were obtained from the gel filtration column. This represented an overall recovery of 33% and a greater than 70 000-fold purification. Furthermore, among the gel filtration fractions collected, fraction 35 contained a total of 5.7×10^7 units and 0.23 mg of protein or a specific activity of 2.5×10^8 units/mg. This fraction represented a recovery of 18% and a greater than 300 000-fold purification.

An analysis of this purification procedure is shown in Figure 3A. To demonstrate the relative proportion of proteins, each lane was loaded with an approximately equivalent amount of protein. The crude starting material (lane 1) had one major band at 67 kDa (presumably bovine serum albumin) and four minor bands with molecular weights of ≥94K, 74K, 70K, and 15K. Material which had been purified by the first three sequential steps of the procedure (lane 2) had four major bands at 87, 74, 58, and 52 kDa. There also appeared to be five minor bands with molecular weights of ≥94K, 38K, 30K, 26K, and 22K. Preparations derived from the CM-BGA column (lane 4) contained one major band at 26 kDa and several relatively minor bands at ≥94, 74, 68, 67, 58, 54, 45, 38, 35, 30, 22, and 15 kDa.

The final product (i.e., gel filtration purified, lane 3) was also scanned in a spectrophotometer as shown in Figure 3B. One major protein was observed at 26 kDa. Four relatively minor proteins, with molecular weights of 74K, 67K, 56K, and 22K, were also witnessed.

Immunoabsorbent Chromatography of HuIFN-γ. As the final product consisted of only one major band (Figure 3A, lane 3, and Figure 3B) and its specific activity (2.5×10^8 units/mg, Table I) closely approximated values previously reported for HuIFN-α (Rubinstein et al., 1979; Zoon et al., 1979) and HuIFN-β (Knight, 1976; Tan et al., 1979; Friesen et al., 1981), it appeared that HuIFN-γ had been purified to essential homogeneity. To verify this conclusion, the purified material was radiolabeled and applied to immunoabsorbent columns containing either anti-HuIFN-γ monoclonal antibodies (I-57 and I-22) or anti-theophylline monoclonal antibodies (I–T). As shown in Figure 4 (lane 7), the starting material (although not easily discernible) consisted of trace amounts of the high molecular weight forms (i.e., 67K and

Table I: Purification of HuIFN-γ

step	total units	total protein (mg)	sp act. (units/mg)	degree of purification (x-fold)	overall recovery (%)
crude	3.2×10^8	3.84×10^5	8.3×10^2		
CPG					
Con A/S	3.0×10^8	57.6	5.2×10^6	6265	94
H/S					
CM-BGA	9.8×10^7	3.4	2.9×10^7	34939	31
Aca 54					
overall[a]	1.0×10^8	1.69	5.9×10^7	71084	33
fraction[b] 34	9.2×10^6	0.86	1.0×10^7	12048	3
fraction 35	5.7×10^7	0.23	2.5×10^8	301204	18
fraction 36	2.9×10^7	0.31	9.3×10^7	112048	9
fraction 37	9.2×10^6	0.29	3.1×10^7	37349	3

[a] The results of the total activity and protein recovered from the gel filtration step. [b] These represent the four fractions of the gel filtration step which contained the majority of the recovered bioactivity.

58K; the 74K moiety appeared only upon overexposure) and the two low molecular weight forms (the 26K and 22K forms appear as one broad band). When applied to either I-57 (lane 1) or I-22 (lane 3), the high molecular weight species passed unbound. The two low molecular weight forms were bound and subsequently eluted from both immunoabsorbents (I-57 in lane 2 and I-22 in lane 4). Nearly all of the starting material passed unbound when applied to the I-T column (lane 5). There existed, however, a small amount of material (approximately 10%) which nonspecifically bound to the resin and was subsequently eluted (lane 6).

These results suggested that both the 26K and the 22K molecular weight species are HuIFN-γ. Furthermore, calculation of the areas under the peaks in Figure 3B revealed that the final product was between 90% and 92% HuIFN-γ.

Amino Acid Analysis of HuIFN-γ. To verify that the 26- and 22-kDa species are HuIFN-γ, SDS-PAGE gel bands were excised and analyzed for amino acid composition. The comparison between these two species and the predicted values derived from the known HuIFN-γ gene (Devos et al., 1982; Gray et al., 1982) is shown in Table II. There was a close correlation between the compositions of the 26-kDa species and the 22-kDa species. In addition, comparisons between the compositions of the 26-kDa moiety, the 22-kDa moiety, and the predicted values derived from the known gene were also similar.

Partial Amino Acid Sequence of HuIFN-γ. A comparison of the predicted sequence analyses is shown in Table III. The results indicate that both the 26-kDa species and the 22-kDa species are HuIFN-γ. Residues 121–126 and 121–133 of the 26-kDa species were determined by spinning-cup and gas-phase techniques, respectively. Of the 10 residues identified, 10 were identical with the predicted assignments. In addition, residues 121–131 and 121–128 of the 22-kDa species were determined by spinning-cup and gas-phase techniques, respectively. Of the 11 residues identified, 11 residues were the same as the predicted assignments.

Discussion

The utility of a sequential chromatographic technique which yielded a product characterized as 40% HuIFN-γ has been previously reported (Braude, 1983b). In an effort to further purify the material, some of HuIFN-γ's other biochemical properties were explored. In this report, HuIFN-γ's IEP was determined to be between 9.5 and 9.8 (Figure 1). As there appears to be only one HuIFN-γ gene (Devos et al., 1982; Gray et al., 1982), the discrepancy between this value and another report (Yip et al., 1982) may be attributed either to differences in glycosylation patterns (which seems most likely) or to the experimental technique.

Table II: Amino Acid Composition of HuIFN-γ

amino acid	nucleotide[a]	26-kDa species[b]	22-kDa species[b]
Asx	20	20.4	20.3
Thr	5	5.5	6.2
Ser	11	12.0	11.9
Glx	19	20.4	21.0
Pro	2	3.1	2.2
Gly	5	7.1	7.4
Ala	8	9.8	9.8
Cys	2	ND[c]	ND
Val	8	5.4	7.0
Met	4	2.4	1.4
Ile	7	6.2	5.7
Leu	10	13.2	12.9
Tyr	5	2.4	2.8
Phe	10	7.5	8.9
His	2	3.5	2.7
Lys	20	19.0	18.1
Arg	7	4.6	4.8
Trp	1	ND	ND

[a] These values were derived from the known nucleotide sequence for HuIFN-γ published by Devos et al. (1982) and Gray et al. (1982). [b] These compositions were computed on the basis of the known nucleotide sequence of 146 residues and, since neither Cys nor Trp was detected, adjusted for 143 residues. [c] ND, not determined.

Advantage was taken of HuIFN-γ's unusually high IEP by including a cationic exchange chromatographic step in the overall purification procedure. Its position in the purification procedure (Figure 2 and Table I) was advantageously placed between the sequential purification steps (i.e., CPG, Con A/S, and H/S) and gel filtration. Consequently, small volumes (derived from the H/S column) could be readily dialyzed and conveniently and rapidly applied to the CM-BGA resin.

Other modifications to the original procedure were the H/S equilibration buffer and the gel filtration resin. The first alteration was lowering the ionic strength of the H/S equilibration buffer so that HuIFN-γ breakthroughs were minimized. The second change was the substitution of Ultrogel AcA 54 for Bio-Gel P-100. Although the P-100 resin gave superior resolution, the additional CM-BGA step sufficiently reduced the protein load so that a resin with superior flow rates (3.8 mL cm^{-2} h^{-1} for AcA 54 vs. 1.9 mL cm^{-2} h^{-1} for P-100) and comparable purification could be employed.

This purification procedure was also shown to be extremely efficient (Table I) and to yield essentially homogeneous HuIFN-γ (see below). Overall, a final product with a specific activity of 5.9×10^7 units/mg (which represented a greater than 70000-fold purification) could be achieved, and with yields of greater than 33%. More importantly however, among the gel filtration fractions collected, one fraction (number 35) had a specific activity of 2.5×10^8 units/mg (which represented a greater than 300000-fold purification) with a yield

Table III: Partial Amino Acid Sequence of HuIFN-γ

position[a]	121	122	123	124	125	126	127	128	129	130	131	132	133
nucleotide[b]	Ala	Glu	Leu	Ser	Pro	Ala	Ala	Lys	Thr	Gly	Lys	Arg	Lys
26-kDa peptide	Ala	Glu	Leu	X	Pro	Ala	Ala	Lys	Thr	X	Lys	Arg	X
22-kDa peptide	Ala	Glu	Leu	Ser	Pro	Ala	Ala	Lys	Thr	Gly	Lys		

[a] The position number of the amino acid relative to the N-terminus as determined from the nucleotide sequence reported by Devos et al. (1982) and Gray et al. (1982). [b] The predicted amino acid as determined from the nucleotide sequence reported by Devos et al. (1982) and Gray et al. (1982).

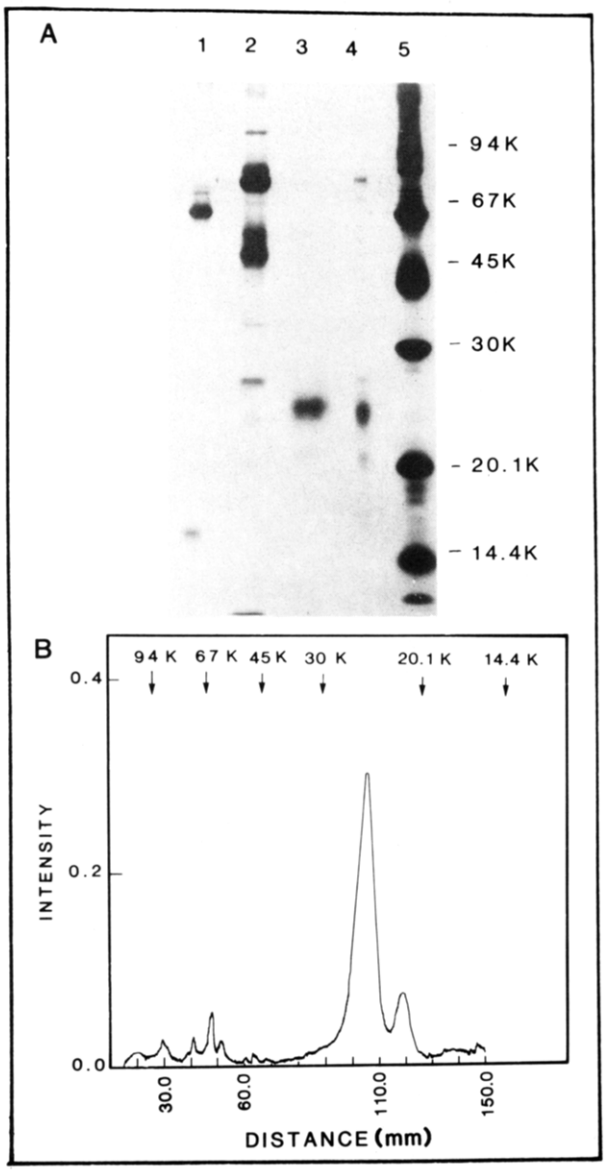

FIGURE 3: SDS–PAGE analysis of purification procedure. Crude HuIFN-γ (lane 1) and sequentially purified (lane 2), CM-BGA purified (lane 4), and gel filtration purified (lane 3) material were electrophoresed on a 12% gel system and Coomassie stained (A). The molecular weight markers (lane 5) were phosphorylase *b* (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and α-lactalbumin (14.4K). Gel filtration purified HuIFN-γ (fraction 35) was also spectrophotometrically scanned (B).

of greater than 17%. When this material was analyzed by SDS–PAGE (Figure 3B), only one major (26 kDa) and four minor (74, 67, 56, and 22 kDa) bands were observed. Samples analyzed in the presence of 2-mercaptoethanol displayed a similar electrophoretic pattern (data not shown). Although not rigorously determined, the apparent molecular weight of HuIFN-γ on the Ultrogel AcA 54 column was similar to the value reported on the Bio-Gel P-100 column (data not shown; Braude, 1983b). The apparent discrepancy between the values determined by gel filtration (35–45 kDa) and SDS–PAGE (22

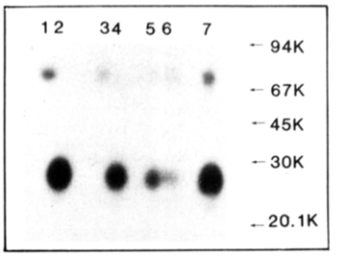

FIGURE 4: Immunoabsorbent chromatography of HuIFN-γ. ^{125}I-Labeled HuIFN-γ (lane 7) was applied to three different immunoabsorbent columns. Two columns (I-57, lanes 1 and 2; I-22, lanes 3 and 4) contained anti-HuIFN-γ monoclonal antibodies. The third column (I-T, lanes 5 and 6) contained anti-theophylline monoclonal antibodies. Peak fractions from both breakthrough (lanes 1, 3, and 5) and bound (lanes 2, 4, and 6) fractions were analyzed by autoradiography of SDS–PAGE. The molecular weight markers were as described above.

and 26 kDa) most likely may be attributed to oligomerization of the protein under nondenaturing conditions (Pestka et al., 1983).

Experiments employing the use of monoclonal antibodies directed against HuIFN-γ (Figure 4) clearly show that the 26K and 22K molecular weight forms observed in Figure 3B are HuIFN-γ. This is in close agreement with the values determined by Western hybridization techniques (Hochkeppel & deLey, 1982) and by measuring residual bioactivities derived from SDS–PAGE (Yip et al., 1982). The smear beteen the 26- and 22-kDa proteins could be resolved into two discreet bands upon shorter exposure times (data not shown), however at the detriment of not observing the high molecular weight bands. Tentatively, the 74K, 67K, and 56K molecular weight forms are considered trace contaminants. It is possible, however, that some of these proteins are HuIFN-γ oligomers which are not recognized by either of the anti-HuIFN-γ monoclonal antibodies.

Assuming that HuIFN-γ resides as both 26K and 22K molecular weight forms, then the area under the curves shown in Figure 3B suggests that the final product (fraction 35) is between 90% and 92% HuIFN-γ. The results also indicate that the ratio of the 26- to the 22-kDa moieties is approximately 10 to 1. Furthermore, as the specific activity of the material was 2.5×10^8 units/mg, the specific activity of pure HuIFN-γ is between 2.7×10^8 and 2.8×10^8 units/mg. This value is very similar to the specific activities previously reported for HuIFN-α (Rubinstein et al., 1979; Zoon et al., 1979) and HuIFN-β (Knight, 1976; Tan et al., 1979; Friesen et al., 1981).

Having demonstrated immunochemically that the 26K and 22K molecular weight forms were HuIFN-γ, the two bands were excised from SDS–PAGE gels and subjected to amino acid composition and sequence analysis. Despite the inherent variability of compositional analyses, there existed a close correlation between the values obtained for the 26- and 22-kDa proteins (Table II). Furthermore, these numbers were in close agreement with the predicted values obtained from the known HuIFN-γ gene (Devos et al., 1982; Gray et al., 1982). These data suggest that both proteins were similar, if not identical.

The limited sequence information obtained on these two species (Table III) adds further evidence that both molecular

weight forms may be the same and that they are most likely identical with the only known HuIFN-γ gene (Devos et al., 1982; Gray et al., 1982). Furthermore, the molecular weights for HuIFN-γ cDNA gene products expressed in mammalian cells (Haynes & Weismann, 1983; Scahill et al., 1983) were very similar to the 26- and 22-kDa values reported herein. Taken together, this information definitely demonstrates (at both the DNA and protein levels) that (i) HuIFN-γ exists as only one protein and (ii) this protein can assume at least two different molecular weight forms.

Lastly, results derived from a series of sequential lectin affinity chromatographic techniques suggest that the difference between these two molecular weight forms can be attributed to variations in glycosylation patterns. Furthermore, it appears that the 22-kDa moeity may be processed from the 26-kDa moiety (Braude & Mehlman, 1983).

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