Purification and Characterization of Recombinant Human Leukocyte Interferon (IFLrA) with Monoclonal Antibodies*

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Recombinant human leukocyte interferon produced in bacteria (IFLrA) was purified to homogeneity with the use of monoclonal antibodies against leukocyte interferon. The purified interferon exhibited a single band of M=19,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid analysis and the NH₂-terminal sequence were consistent with the sequence predicted from the DNA. Some of the purified product contained NH₂-terminal methionine; the terminal methionine was removed from the rest of the chains.

We have isolated and identified DNA recombinants containing sequences for human leukocyte and fibroblast interferons (1). One such clone was used to isolate a full length cDNA recombinant that was reconstructed to express a human leukocyte interferon (IFLrA) in bacteria (2). With the isolation of 13 monoclonal antibodies to human leukocyte interferon (3), it was one of our goals to use one or more of them for the purification of the interferon produced in bacteria. This purification as well as characterization of the bacterial product is described here.

EXPERIMENTAL PROCEDURES

Bacterial Fermentation—Overnight cultures of Escherichia coli 294 containing recombinant plasmid IFLrA (2) were grown in LB broth (4). One liter of the overnight culture was diluted to 10 liters with minimal M-9 medium with casamino acids in the absence of tryptophan to induce the interferon protein that is controlled by the tryptophan regulatory region. Bacteria were harvested during late logarithmic growth by centrifugation and the bacterial pellets were stored at -20°C until used. All fermentations and procedures were performed in accordance with the National Institutes of Health Guidelines for Recombinant DNA Research.

Preparation and Purification of Monoclonal Antibodies from Ascitic Fluid—Five female BALB/c mice were each inoculated with 5 to 10 x 10⁶ hybridoma cells from midlog growth phase. About 2 x 10⁸ viable cells obtained from the mouse which produced fluid were inoculated intraperitoneally into each of 10 or more mice. The ascitic fluid was collected repeatedly (2 to 4 times) from each mouse. Up to three transfers and collections may be performed from one group of mice to the next. Ascitic fluid from mice at each transfer was pooled.

Cells and debris were removed from the ascitic fluid by low speed centrifugation (500-1,000 x g) for 15 min. Then centrifugation was performed for 90 min at 18,000 rpm in the SS34 Sorvall rotor without braking. The supernatant was frozen and stored at -20°C. After thawing, additional fibrin and particulate material were removed by centrifugation at 35,000 rpm for 90 min in the Spinco Type 35 rotor.

Batches of ascitic fluid from each transfer were tested for specific antibody activity by a solid phase antibody-binding assay (3) and pooled if found satisfactory.

Concentration of protein in the pooled solutions was estimated by the approximation that 1 mg of protein yields an absorbance of 1.2 at 280 nm in a cuvette with a path length of 1.0 cm. Ascitic fluids with high levels of antibody contained 30-35 mg of protein/ml. This is equivalent to 4-7 mg of IFLrA interferon/ml. The fluid eluted with phosphate-buffered saline (0.01 M sodium phosphate, pH 7.3, 0.15 M NaCl) to a protein concentration of 10 to 12 mg/ml (12 to 15 A₂₆₀ units/ml).

To each 100 ml of diluted solution, 90 ml of room temperature saturated ammonium sulfate solution were added slowly with vigorous stirring to 0.0°C. The suspension was kept at ice for 40 to 60 min and then centrifuged for 15 min at 10,000 rpm in a Sorvall GS-A rotor at 4°C. The supernatant was decanted and drained well. The protein pellets were dissolved in 0.02 M Tris-HCl (pH 7.9)/0.04 M NaCl (Buffer A; about 5 ml/250-ml centrifuge bottle). The protein solution was dialyzed for 16 to 18 h at room temperature against 100 volumes of Buffer A with at least one change of the buffer. The dialyzed solution was centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 10 min to remove undissolved material. About 30 to 35% of the original amount of total protein in the ascitic fluid was recovered as estimated by absorption at 280 nm.

The solution containing 30 to 40 mg of protein/ml was then applied to a column of DEAE-cellulose (DE52, Whatman) equilibrated with Buffer A. A column bed volume of at least 100 ml was used for each gram of protein applied. The antibody was eluted from the column with a linear NaCl gradient containing 0.02 M Tris-HCl, pH 7.9, from 0.04 to 0.5 M NaCl. Pooled peak fractions eluting between 0.06 and 0.1 M NaCl were concentrated by precipitation with an equal volume of saturated ammonium sulfate and centrifugation. The protein pellets were dissolved in 0.2 M NaHCO₃ (pH 8.0)/0.3 M NaCl (Buffer B) followed by dialysis against three changes of the same buffer at room temperature. The dialyzed solutions were centrifuged at 20,000 x g for 15 min to remove any insoluble material. Protein concentration was adjusted to 20 to 25 mg/ml with Buffer B. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of representative monoclonal antibodies is shown in Fig. 1.

Preparation of Immunoadsorbents—Affi-Gel 10 (Bio-Rad) was washed on a sintered glass filter three times with ice-cold isopropanol followed by three washes with ice-cold distilled water. The gel slurry (~50% in cold water) was transferred to plastic tubes and sedimented by a brief centrifugation. The supernatant was aspirated. The packed gel was mixed with an equal volume of purified antibody solution and rotated end over end at 4°C for 5 h. After rotation, the gel was centrifuged and then washed twice with Buffer C (0.1 M NaHCO₃/0.15 M NaCl) to remove uncoupled antibody. Protein determination of the combined washes revealed that more than 90% of antibody was coupled to the gel.

To block unreacted sites, the gel was mixed with an equal volume of 1 M Tris-HCl, pH 8.0 and rotated end over end at room temperature for 60 min. The gel slurry was washed free of reactants with phosphate-buffered saline and stored in phosphate-buffered saline in the presence of 0.02% (w/v) sodium azide at 4°C.

Purification of Recombinant Human Leukocyte Interferon (IFLrA)—All purification steps were carried out at 4°C. Frozen cells (1 kg) were suspended in three volumes (3 liters) of lysis buffer (0.1 M Tris-HCl, pH 7.9, 10% sucrose, 5 mM EDTA, 0.2 M NaCl, 10 µM Xg)

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Purification of Recombinant Leukocyte Interferon

Purification of monoclonal antibodies LI-3, LI-5, LI-6, LI-7, and LI-8. Monoclonal antibodies were reduced and electrophoresed as described (5). Molecular weight standards (STDS) were as follows: 95,000, phosphorylase A from rabbit muscle; 67,000, bovine serum albumin; 60,000, catalase; 45,000, ovalbumin; 40,000, aldolase from rabbit muscle; 36,000, glyceraldehyde-3-phosphate dehydrogenase from yeast; 25,000, chymotrypsinogen.

phenylmethylsulfonyl fluoride). The suspension was passed once at 6,000 p.s.i. and once at 600 p.s.i. through the Manton-Gaulin Press. Polymylin P (pH 7.9) was added to the lysate to a final concentration of 0.5% (w/v). The mixture was stirred for 1 h and then centrifuged for 30 min at 7,500 rpm (Sorvall GS-3 rotor). Solid ammonium sulfate was added to the supernatant to 30% saturation. The solution was then centrifuged (7,500 rpm, 30 min), any pellet was discarded, and the supernatant was brought to 65% saturation with solid ammonium sulfate. After centrifugation (7,500 rpm, 30 min), the pellet was suspended in a small volume of Buffer D (25 mM Tris-HCl, 0.01% phenylmethylsulfonyl fluoride) and dialyzed against five changes of the same buffer. The suspension obtained was centrifuged (Sorvall GS-3 rotor; 10,000 rpm, 15 min) and, after addition of NaCl to 0.15 M, the supernatant was loaded at 50 ml/h onto an immunoadsorbent column (2.5 x 3.5 cm; 17-ml bed volume; 408 mg of purified monoclonal antibody LI-8) equilibrated with Buffer E (0.2 g/liter of KCl, 0.2 g/liter of KH$_2$PO$_4$, 0.1% Triton X-100, pH 2.5). Interferon activity was eluted in a 1-propanol gradient in 1 M ammonium sulfate fraction (fractions 30-80), Buffer F (fractions 81-116), Buffer G (fractions 117-124), and Buffer H (fractions 125-140). Fractions (127-130) containing interferon activity were pooled. Almost all the proteins applied to the column were found in the flow-through fraction. Interferon was measured by radioimmunoassay (8). One radioimmunoassay unit corresponds to about 0.7 antiviral unit.

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate fraction</td>
<td>700</td>
<td>37,100</td>
<td>7.4 x 10$^6$</td>
<td>2.0 x 10$^5$</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Antibody column pool</td>
<td>27</td>
<td>30</td>
<td>7.0 x 10$^6$</td>
<td>2.3 x 10$^5$</td>
<td>1.15</td>
<td>95</td>
</tr>
<tr>
<td>CMS2</td>
<td>30</td>
<td>20</td>
<td>6.0 x 10$^6$</td>
<td>3.0 x 10$^5$</td>
<td>1.50</td>
<td>81</td>
</tr>
</tbody>
</table>

Purification of interferon by monoclonal antibody immunoabsorbant column chromatography. Experimental details are described in the text. The column (monoclonal antibody LI-8 attached to Affi-Gel 10) was washed sequentially with Buffer D (fractions 30-80), Buffer F (fractions 81-116), Buffer G (fractions 117-124), and Buffer H (fractions 125-140). Fractions (127-130) containing interferon activity were pooled. Almost all the proteins applied to the column were found in the flow-through fraction. Interferon was measured by radioimmunoassay (8). One radioimmunoassay unit corresponds to about 0.7 antiviral unit.

**FIG. 2.** Purification of interferon by monoclonal antibody immunoabsorbent column chromatography. Protein was determined by the method of Lowry et al. (6) with crystalline bovine serum albumin as standard. Interferon activity was determined by a cytopathic effect inhibition assay with vesicular stomatitis virus and MDBK cells (bovine kidney cell line) as described (9).

**FIG. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of monoclonal antibodies LI-3, LI-5, LI-6, LI-7, and LI-8. Molecular weight standards (STDS) were as follows: 95,000, phosphorylase A from rabbit muscle; 67,000, bovine serum albumin; 60,000, catalase; 45,000, ovalbumin; 40,000, aldolase from rabbit muscle; 36,000, glyceraldehyde-3-phosphate dehydrogenase from yeast; 25,000, chymotrypsinogen.
**Amino Acid Analysis**—Samples of the purified interferon were hydrolyzed, and amino acid analysis was performed with fluorescamine as previously described (11). Tryptophan was measured after hydrolysis in 6 N HCl containing 4% thioglycolic acid. Part of the sample was reduced and carboxymethylated before analysis.

**Amino Acid Sequencing**—Automatic Edman degradations were performed in a modified (12-14) Beckman sequenator as described (15).

**Analytical Isoelectric Focusing**—Isoelectric focusing was performed on a 3% cross-linked polyacrylamide thin layer plate (9 × 24 cm) in a pH 3.5–9.5 gradient containing LKB ampholines (1 M H3PO4 anode-electrode solution; 1 M NaOH cathode-electrode solution) at 10 °C, 30 watts/plate. After 1.5 h, the gel was fixed in 57.5 g of trichloroacetic acid/17.25 g of sulfosalicylic acid/500 ml of H2O for 0.5–1 h, washed in destaining solution (ethanol/acetate/H2O, 25:86, v/v) for 5 min, stained for 10 min at 60 °C in 0.46 g of Coomasie brilliant blue R-250 in 400 ml of destaining solution, and then destained for 15 h at room temperature.

**RESULTS**

The monoclonal antibody columns provided major purification of interferon in a single step. Most of the proteins passed directly through the column. Interferon was eluted by the pH 2.5 buffer (Fig. 2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions 87 to 140 (Fig. 3) demonstrated that interferon, barely visible in the 0–65% ammonium sulfate fraction, was almost homogeneous after a single passage of the ammonium sulfate fraction through the monoclonal antibody column. In fact, fractions 131 and 132 appeared to represent almost homogeneous interferon. The major protein contaminating the interferon after the monoclonal antibody column exhibited $M_r = 45,000$ and possibly was bacterial elongation factor EF-Tu or a dimer of ISLrA (Figs. 3 and 4). After the carboxymethyl-cellulose step, the interferon appeared to be homogeneous. No additional bands were detectable after loading of the gel with up to 20 μg of the purified interferon (Fig. 4). The purification is summarized in Table I. In addition, it can be seen that a number of the monoclonal antibodies (LI-3, LI-5, LI-6, LI-7, LI-8, and LI-9) were effective in purification of IFILrA (Fig. 4).

The amino acid composition of IFILrA was consistent with that predicted from the DNA sequence (Table II). A portion of the IFILrA was reduced and carboxymethylated in order to obtain a reliable estimate of the cysteine content. Since this precludes measurement of tryptophan, a sample was also hydrolyzed without carboxymethylation for analysis. Both determinations are shown.

An interferon sample, taken after chromatography on the monoclonal antibody column but before chromatography on carboxymethyl-cellulose, was subjected to high performance liquid chromatography on a reverse phase RP-8 column at pH 4.0 (Fig. 5). The major protein band elutes at 33% 1-propanol and corresponds to the peak of interferon activity. Thus, even before the last purification step, the interferon preparation contains only minor contaminants.

Interferon (20 μg) purified through the final carboxymethyl-cellulose chromatography was subjected to isoelectric focusing (Fig. 6). The major protein is a closely spaced doublet, migrating with a pI of 6.2–6.3.

Ten nanomoles of reduced and carboxymethylated protein underwent Edman degradation in an automatic sequenator. The amino acid sequence of the purified protein was in accord with that predicted by the DNA sequence. Two chains were evident. Some of the chains contained the initiator methionine; methionine was removed from the rest of the chains. Hydrazinolysis of 1 nmol of IFILrA for 5 h at 105 °C released a single amino acid, glutamic acid, with a yield of 50%. This result provides identification of the COOH-terminal residue as glutamic acid as predicted by the DNA sequence.

IFILrA (400 pmol) was digested with trypsin and the resulting peptides were analyzed by high performance liquid chromatography (Fig. 7). Seventeen of the expected 21 peptides were separated, although some of these may be overlapping if digestion was not complete. The profile is very similar to those observed for leukocyte interferon species isolated from leukocytes of patients with chronic myelogenous leukemia (16).

Antiviral activity of IFILrA was about the same on human and bovine cells (Table III). Little or no activity was demonstrated with mouse cells.

**DISCUSSION**

The purification of recombinant human leukocyte interferon A (IFILrA) can be carried out simply, efficiently, and rapidly with the use of monoclonal antibodies to human

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**Fig. 3**. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoabsorbent column fractions. Ten microliters of each fraction were subjected to electrophoresis according to the procedure of Laemmli (5) on a linear 7.5 to 15% polyacrylamide gradient gel containing 0.1% sodium dodecyl sulfate. The gel was stained for 1 h at 37 °C with 0.25% Coomasie brilliant blue in methanol/acetate/water (5:1:5) and destained with 5% methanol in 7.5% acetic acid. Protein standard markers were purchased from Bethesda Research Laboratories: 200,000, myosin heavy chain; 92,500, phosphorylase b from rabbit muscle; 68,000, bovine serum albumin; 43,000, ovalbumin; 26,000, α-chymotrypsinogen; 18,400, β-lactoglobulin; 12,300, cytotoxic c. STDS, standards; AS, ammonium sulfate; HAc, acetic acid.
Purification of Recombinant Leukocyte Interferon

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified interferon. Experimental details were as described in Fig. 3. Approximately 20 μg of interferon purified by various monoclonal antibody immunoabsorbent columns (LI-3, LI-5, LI-6, LI-7, and LI-8) were subjected to electrophoresis. LI-8(#2), LI-8(#3), and LI-8(#4) represent the second, third, and fourth passages of an ammonium sulfate fraction through the same LI-8 column. The first passage through the LI-8 column is shown in Figs. 2 and 3. The interferon peak from the first LI-8 column (Figs. 2 and 3) was pooled and further purified by carboxymethyl-cellulose (CM52) chromatography (see text). Samples from various steps of the CM52 chromatography are shown here: CM52 FT, flow-through; CM52 0.1 M, 0.1 M ammonium acetate wash; CM52 0.5 M, 0.5 M ammonium acetate elution of interferon from the column. The CM52 0.5 M sample represents 20 μg of protein. Molecular weight standards are as in Fig. 3.

Table II

Amino acid composition of recombinant human leukocyte interferon A (IFLrA)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>No. of residues/molecule</th>
<th>Residues predicted from DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>11.6</td>
<td>I, II*</td>
</tr>
<tr>
<td>Ser</td>
<td>14.2</td>
<td>I, II*</td>
</tr>
<tr>
<td>Thr</td>
<td>9.7</td>
<td>I, II*</td>
</tr>
<tr>
<td>Glx</td>
<td>26.4</td>
<td>I, II*</td>
</tr>
<tr>
<td>Pro</td>
<td>4.1</td>
<td>I, II*</td>
</tr>
<tr>
<td>Gly</td>
<td>5.4</td>
<td>I, II*</td>
</tr>
<tr>
<td>Ala</td>
<td>8.1</td>
<td>I, II*</td>
</tr>
<tr>
<td>Val</td>
<td>6.1</td>
<td>I, II*</td>
</tr>
<tr>
<td>Met</td>
<td>5.0</td>
<td>I, II*</td>
</tr>
<tr>
<td>Ile</td>
<td>7.4</td>
<td>I, II*</td>
</tr>
<tr>
<td>Leu</td>
<td>20.6</td>
<td>I, II*</td>
</tr>
<tr>
<td>Tyr</td>
<td>5.0</td>
<td>I, II*</td>
</tr>
<tr>
<td>Phe</td>
<td>9.7</td>
<td>I, II*</td>
</tr>
<tr>
<td>His</td>
<td>2.8</td>
<td>I, II*</td>
</tr>
<tr>
<td>Lys</td>
<td>10.6</td>
<td>I, II*</td>
</tr>
<tr>
<td>Arg</td>
<td>9.2</td>
<td>I, II*</td>
</tr>
<tr>
<td>Cys</td>
<td>7.8</td>
<td>I, II*</td>
</tr>
<tr>
<td>Cys (Cm)</td>
<td>1.5</td>
<td>ND*</td>
</tr>
<tr>
<td>Trp</td>
<td>1.5</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Analysis II was performed on reduced and carboxymethylated IFLrA, in order to more accurately determine Cys content.
ND, not determined.

leukocyte interferon. The purified protein is homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and yields an amino acid composition and sequence expected from the DNA sequence. The specific activity of homogeneous IFLrA is comparable to that of the homogeneous natural human leukocyte interferon (16, 17). The purified product consists of two chains, most with and some without the NH2-terminal methionine. At least one E. coli protein, initiation factor IF-3, has been found to be present with and without the terminal methionine (12).

Several of the monoclonal antibodies could be used to purify IFLrA. Since repeated use of the monoclonal antibody columns is possible, these affinity columns provide a convenient method for preparing homogeneous human leukocyte interferon from bacterial fermentations. Interferon prepared by modifications of the procedures described herein is now being used in clinical trials in humans. It should be noted that modifications of these procedures were made in order to

Fig. 5. High performance liquid chromatography of monoclonal antibody-purified interferon. Approximately 45 μg of interferon purified with the monoclonal antibody immunosorbent column LI-8 were chromatographed on a reverse phase RP-8 column at pH 4.0 as described under "Experimental Procedures."

Fig. 6. Isoelectric focusing of purified interferon. About 20 μg of interferon purified through carboxymethyl-cellulose chromatography was subjected to analytical electrofocusing as described under "Experimental Procedures." Interferon has a pI of 6.2-6.3.
Antiviral activity of recombinant human leukocyte interferon A (IFLrA) on human, bovine, and mouse cells

Repeated assay of IFLrA on MDBK, WISH (human), and AG-1732 cells yielded the following ratios of the antiviral activity: the mean MDBK/AG-1732 activity ratio was 1.7 with S.D. = 0.22 for seven determinations; the mean MDBK/AG-1732 activity ratio was 1.6 with S.D. = 0.51 for four determinations.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Relative titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG-1732 (human)</td>
<td>100</td>
</tr>
<tr>
<td>MDBK (bovine)</td>
<td>160</td>
</tr>
<tr>
<td>L-929 (mouse)</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Fig. 7. Tryptic profile of purified interferon. IFLrA (400 pmol) was digested with trypsin and the peptides were analyzed as described under "Experimental Procedures."

Table III

obtain a product suitable for parenteral use in humans. In addition, several biological activities of this purified recombinant interferon have been determined. The recombinant IFLrA exhibits antiviral activity and antiproliferative activity (18) comparable to crude and purified natural leukocyte interferons. IFLrA also stimulates natural killer-cell activity and inhibits hemopoietic colony formation. With the eventual availability of large amounts of homogeneous IFLrA, extensive clinical trials, biological studies, and determination of structure by x-ray crystallographic methods are achievable.

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REFERENCES
