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Human Fibroblastoid Interferon: Immunosorbent Column Chromatography and N-Terminal Amino Acid Sequence†

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ABSTRACT: Three mice and one rabbit were inoculated with purified human fibroblastoid interferon. Neutralizing activity to human fibroblastoid interferon was observed in the serum of these animals with the rabbit showing the highest anti-interferon titers (10^6 neutralizing units/mL). Rabbit antiserum was coupled to cyanogen bromide activated Sepharose, and the resulting material was tested for use in the purification of human fibroblastoid interferon. Pure interferon obtained by this procedure was analyzed, and we report the sequence of the first 13 N-terminal amino acid residues of this protein.

In order to determine the primary structure of a scarce protein like human interferon, it is necessary to fulfil a few requirements: to maximize the cellular production of the protein (Tan et al., 1970; Ho et al., 1973; Zoon et al., 1980; Cantell & Hirvonen, 1977), to increase the sensitivity of amino acid sequence methodologies (Reinbolt et al., 1977; Hunkapiller & Hood, 1978, 1980; Wittmann-Liebold et al., 1977), and to increase the efficiency of the purification of the protein (Tan et al., 1979, 1980; Berthold et al., 1978; Knight, 1976; Knight et al., 1980; Edy et al., 1976; Davey et al., 1974; Len et al., 1978; Zoon et al., 1979; Rubinstein et al., 1978, 1979). Much effort has been made in the cellular production of interferon, and rapid advances have been made in the area of microsequencing proteins at the subnanomole to nanomole range (Hunkapiller & Hood, 1980). However, the available procedures for isolating pure interferon from serum containing a crude interferon preparation provide a yield of 1–10% of the pure protein. In this paper we report an efficient method using an immunosorbent column for the purification of human fibroblastoid interferon which provides a recovery of 30% or more pure interferon from a serum-containing preparation and 70% when the interferon is prepared in serum-free medium. The new procedure has produced preparations (400–1000 pmol) of pure interferon for its amino acid sequence analysis.

Experimental Procedures

Interferon Production and Assay. Interferon was produced and concentrated from a C-10 cell line as previously described in serum-containing or serum-free medium (Tan et al., 1979) and assayed on human fibroblasts according to a semimicro-method (Tan, 1975). Titers were determined by visual assessment of the viral cytopathic effect. A human fibroblast preparation referenced to 69/19 MRC human leukocyte interferon was used as the interferon standard. This standard was kindly tested and supplied by Dr. W. Merk and Dr. G. Bodo.

Protein for Inoculation. Pure interferon for inoculation was prepared by the method of Tan et al. (1979) and Berthold et al. (1978). The purity of interferon was verified by analytical slab polyacrylamide gel electrophoresis (Figure 1).

Generation of Antibodies. Three BALB/c mice were each inoculated with 10–20 μg of the purified interferon preparation weekly for 3 months. The purified antigen was mixed 1:1 v/v with Freund's adjuvant and injected intraperitoneally. One New Zealand White rabbit was inoculated with the same amount of antigen on a regimen similar to that for the mouse, except that in the first eight inoculations Freund's adjuvant was omitted and the routes of inoculation were different as indicated in Fugure 2. Test sera were obtained from the animals once weekly thereafter.

For determination of interferon neutralizing activity, an aliquot of the test serum diluted 100-fold was incubated with an equal volume of human interferon. After the incubation, the mixture was assayed for antiviral activity as described above. The neutralizing titer was obtained by first finding the well in which half the assay cells were protected; the value of the original interferon activity in that well was then multiplied by 200, the serum dilution factor. For example, if 50% protection occurred in a well containing 1×10^3 units of antiviral activity (prior to incubation), then the neutralizing titer of that serum would be 2×10^5 units/mL. Antiviral units were standardized as described above.

Preparation of Immunosorbent Column. Rabbit antiserum was treated with 40% ammonium sulfate. The precipitated immunoglobulin fraction was sedimented by centrifugation at

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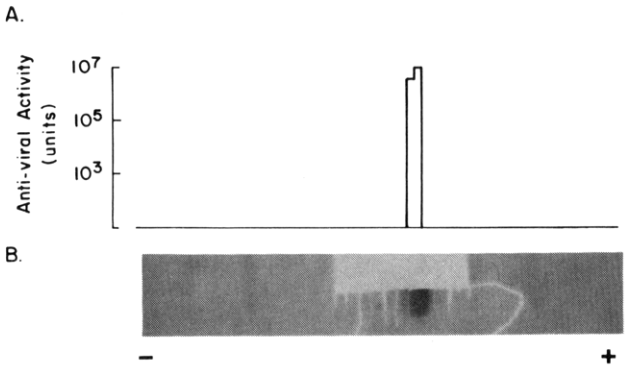

FIGURE 1: Purity of human fibroblastoid (C-10) interferon. The interferon was purified as described before (Berthold et al., 1978) and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (lower panel). A portion of the gel was cut out and processed to determine the location of antiviral activity (upper panel).

10000*g* for 15 min. The precipitate was redissolved in 20 mM sodium phosphate buffer, pH 7.2 (PB), and dialyzed against the same buffer. The partially purified antiserum was then mixed with a slurry of cyanogen bromide activated Sepharose 4B (3 g dry weight, Pharmacia) in 0.1 M sodium bicarbonate, pH 8.3. The mixture was gently stirred overnight at 4 °C, after which it was allowed to stand and the clear supernatant removed. This material was then treated with a 10-volume excess of 0.1 M Tris-HCl, pH 8.3, for 2 h. At the end of this treatment the Sepharose was washed with 0.1 M sodium borate buffer, pH 8.0, containing 1 M NaCl. This washing procedure was repeated 5 times. Thereafter, the Sepharose was washed in PB and stored at 4 °C.

In a separate experiment, Sepharose CL4B was used as the column matrix. In this case it was activated with cyanogen bromide by a modified method of Cuatrecases & Anfinsen (1971) and immediately (within 60 s) mixed with the partially purified antiserum as described above.

Purification by the Immunosorbent Column. Crude interferon was concentrated and partially purified on a controlled-pore glass column first described by Edy et al. (1976). The pH of this material was acidic (100 mM acetic acid); 1 volume of CPG-purified interferon was diluted with 3 volumes of glass-distilled water and 1 volume of glycerol to change the pH of the preparation to neutral before application to the immunosorbent column. After EDTA was added to a final concentration of 0.1 mM and the pH was increased to 5.0 by adding 0.01 volume of 1 M sodium acetate, the preparation was applied to the CM-cellulose column (10-mL bed volume). The column was then eluted with the following solutions, all of which also contained 20% glycerol and 0.1 mM EDTA: (1) 20 mM sodium acetate buffer, pH 5, (2) 3 column volumes of PB, (3) 3 column volumes of PB containing 0.05 M NaCl, (4) 3 column volumes of PB containing 0.075 M NaCl, (5) 3 column volumes of PB containing 0.1 M NaCl, and (6) 3 column volumes of PB containing 0.3 M NaCl. The CM-cellulose purified preparation was diluted 4 times with distilled water and applied at room temperature to the immunosorbent column (10–20-mL bed volume). The column was eluted with the following: (1) 5 column volumes of PB, (2) 5 column volumes of PB containing 0.3 M NaCl, (3) 5 column volumes of 20 mM sodium acetate, pH 5, and (4) 3–4 column volumes of 100 mM acetic acid. The immunosorbent column purified interferon in 100 mM acetic acid was dialyzed against water containing 0.001% $NaDodSO_4$ and lyophilized. This material was then subjected to a final purification step using preparative $NaDodSO_4$–polyacrylamide gel electrophoresis as previously described (Tan et al., 1979). Protein was eluted from the gel in 20 mM Tris–acetate, pH 8.5, containing 1% $NaDodSO_4$ as described below.

Table I: Neutralization of the Antiviral Activity of Different Interferon Preparations by Rabbit Antisera and Mouse Antisera

anti-serum	type of interferon	sp act. (units/mg)	neutralizing act. (units/mL)
rabbit	human leukocyte interferon	1×10^6	0
	human fibroblast interferon	1×10^4	4.15×10^4
	human fibroblastoid interferon	2×10^4	7.2×10^5
	human fibroblastoid interferon	1×10^6	1.72×10^6
	mouse L cell interferon	1×10^4	0
mouse	human leukocyte interferon	1×10^6	0
	human fibroblastoid interferon	1×10^6	0.8×10^5
	mouse L cell interferon	1×10^4	0

Radioamidation of Purified Interferon. Interferon eluted from the polyacrylamide gel was dialyzed against water containing 0.001% $NaDodSO_4$ and then lyophilized. The residue was dissolved in 0.1 M sodium borate, pH 8.5, and radioiodinated with methyl 3,5-diiodohydroxybenzimidate (sp act. 4000 Ci/mmol, Amersham) according to the method of Wood et al. (1975). Alternatively, the preparation was labeled after the method of Bolton & Hunter (1973) as previously described for interferon (Berthold et al., 1978). After the reaction was complete, the radiolabeled preparation was dialyzed extensively against 0.001% $NaDodSO_4$ in water at room temperature.

Sample Preparation for N-Terminal Amino Acid Sequencing. Interferon was electroeluted from the polyacrylamide gel by a method described by Lazarides (1976) with the following modifications. The gels were cut into $2 \times 6 \times 20$ mm slices and placed in a Pasteur pipet which had been previously plugged at the bottom end with a small amount of Kimwipe tissue paper. The protein was electrophoresed out of the gel in a buffer consisting of 0.1% sodium dodecyl sulfate ($NaDodSO_4$ electrophoresis grade from Bio-Rad, CA) containing 0.19 M glycine and 25 mM Tris, pH 8.3, at 5 mA/tube for 16–20 h. The interferon was electroeluted into 10-mm flatwidth dialysis tubing (3 cm long; Spectrapor Spectrum Medical Industry, CA). The interferon was dialyzed in the same dialysis tubing against 3 changes (12 h each) of 0.15 M NaCl containing 0.1% $NaDodSO_4$ and then against 3 changes of 0.02% $NaDodSO_4$ (12 h each) at room temperature. Thereafter, the preparation was lyophilized to dryness in a sterile plastic tube. According to this procedure, it is possible to obtain near-quantitative recovery of interferon from the gel. The pure protein in subnanomole amounts (400–1000 pmol) was used for N-terminal amino acid sequence analyses by the method of Hunkapillar & Hood (1980).

Results and Discussion

Immunological Response and Antibody Specificity. Three BALB/c mice and one New Zealand White rabbit were inoculated with purified interferon according to the schedule described under Experimental Procedures. The interferon neutralizing titers of the antisera reached a maximum of 10^4 and 10^6 units/mL in the mouse and rabbit, respectively (Table I). Whether this difference in antibody levels is due to species differences is not known since the small amount of antigen available limited the number of animals inoculated. As shown in Figure 2, the neutralizing activity of the rabbit antiserum remained at 10^6 units/mL for 2–3 weeks and then decreased by ~50%. However, when the rabbit was reinoculated with 10–20 μg of pure interferon, the maximal neutralizing activity of the antiserum was reestablished.

The specificity of the rabbit and mouse antisera was investigated. The results presented in Table I show that the

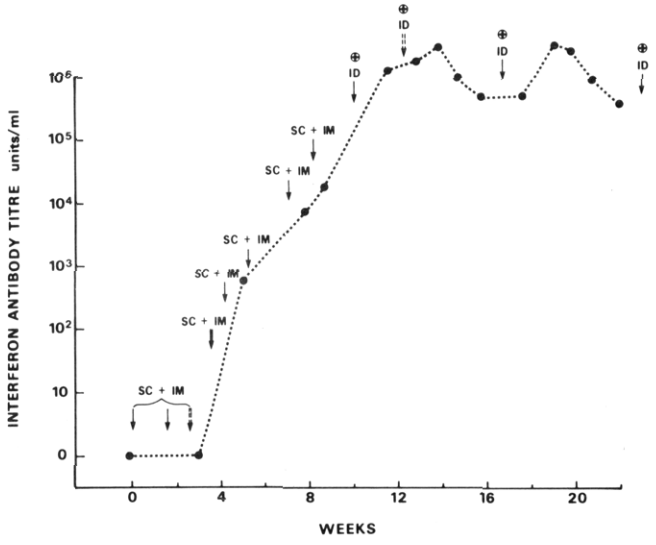

FIGURE 2: Interferon neutralizing activity in the serum of a rabbit inoculated with purified interferon. (↓) 1×10^6 antiviral units; (=⇒) 2×10^6 antiviral units; (⇒) 3×10^6 antiviral units; ⊕ antigen combined with complete Freund's adjuvant.

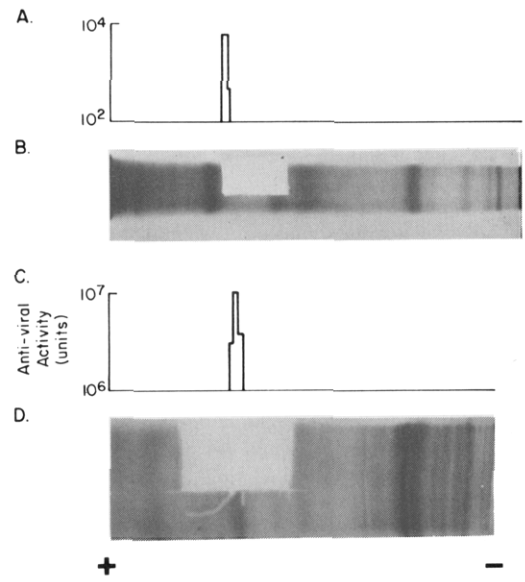

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of partially purified C-10 interferon preparations. (A) Antiviral activity, CM-cellulose purified interferon; (B) stained proteins, CM-cellulose purified interferon; (C) antiviral activity, immunosorbent purified interferon; (D) stained proteins, immunosorbent purified interferon.

neutralizing activity of these antisera was highly specific for human fibroblast type interferon and did not interact with mouse L cell or human leukocyte interferons. Moreover, the rabbit antisera were 10 times more active against human fibroblastoid interferon than against diploid fibroblast interferon. These findings indicate that the antigenic site(s) of various interferons are different. In this regard, it is interesting to note that the production of human interferon has been assigned to more than one chromosome (Slate & Ruddle, 1979; Tan et al., 1974) and a two-gene hypothesis has been proposed based on the specificity of antisera directed against leukocyte and fibroblast interferons (Havell et al., 1975; Vilcek et al., 1977). An alternative explanation for the distinct antigenicities of interferons is that they result from differences in posttranslational modifications of the interferon peptide or that they reflect different regulatory factors controlling transcription of the structural gene. Obviously, the definitive explanation for the observed specificity of various antisera awaits the amino acid sequence analysis of the different interferons, the partial structures of which are now known (Zoon et al., 1980; Knight et al., 1980).

Another property of the rabbit antisera was its slightly higher potency (two- to threefold) against interferon of greater purity, which suggests that impurities in the interferon preparation could interfere with the neutralizing activity of the antiserum. It is known that interferon can bind various protein impurities by hydrophobic and possibly other interactions (Tan et al., 1979; Berthold et al., 1978). Perhaps such nonspecific protein interactions could hinder the optimal binding of interferon with its antibody. On the other hand, these impurities could also compete with interferon for some antibody binding sites.

Recovery. Human interferons from serum-containing preparations have been purified. The recovery is generally in the range of 1–10% and sometimes even less (Tan et al., 1979, 1980; Berthold et al., 1978; Knight, 1976; Knight et al., 1980; Edy et al., 1976; Davey et al., 1974; Len et al., 1978; Zoon et al., 1979; Rubinstein et al., 1978, 1979). A review of these purification procedures indicates the losses occur as the result of many purification steps and during the final stages when the protein concentration is low. We have limited the number of steps in the present purification procedure to four. The introduction of an immunosorbent column in the procedure is largely responsible for the improved overall yield by its ability to concentrate and purify a diluted preparation of a partially purified preparation. In effect, crude interferon was first passed through a CPG column to remove some of the contaminating serum proteins; 95% of the applied antiviral activity was recovered. However, it was necessary to change the pH of the CPG-purified interferon from pH 3 to 7 before it could be applied to the immunoadsorbent column. We have previously reported that this cannot be done by simple titration to the required pH due to precipitation (Tan et al., 1979). To change the pH to neutrality, we have found that it is possible to chromatograph the CPG-purified preparation through a CM-cellulose column at pH 5. At this pH the protein fractions responsible for the precipitation during the pH change are presumably removed from interferon. Interferon can then be eluted from the column at neutral pH by increasing the salt concentration. The impurities of this preparation are shown in Figure 3A. Evidently, at this stage interferon protein is not sufficiently enriched to be stained by Coomassie Brilliant Blue. The presence of glycerol and EDTA is required to improve the recovery of interferon during this step.

When the partially purified CM-cellulose preparation was applied to the immunosorbent column at neutral pH, interferon was strongly bound. In contrast, interferon was not bound to a similar column prepared from control rabbit serum, indicating that the binding of interferon to the immunosorbent column is specific. Antiviral activity could not be eluted from the column by 0.3 M NaCl while a small amount (5%) could be eluted from the column by 0.5 M NaCl. However, 100 mM acetic acid, pH 2.9, effected an excellent recovery (70–95%) of interferon from the column. Interferon eluted from the immunosorbent column was concentrated by lyophilization and the purity of the preparation examined. Analytical $NaDodSO_4$–polyacrylamide gel electrophoresis showed that, in addition to interferon, the preparation contained a large amount of higher molecular weight proteins, some of which were not present in the CM-cellulose preparation (Figure 3). Several questions were raised. Was the antigen impure, was the immunosorbent column behaving like a hydrophobic affinity column to which impurities were bound, could the impurities

Table II: Purification of C-10 Interferon

starting prepn	procedure	sp act. (units/mg)	% recovery from crude
serum-containing crude		1×10^5	100
	controlled-pore glass	5×10^5	90
	CM-cellulose	1×10^6	72
	immunosorbent	1×10^7	57
	$NaDodSO_4$–polyacrylamide gel electrophoresis	2×10^8	31
serum-free crude		4×10^5	100
	immunosorbent	0.7×10^7	95
	$NaDodSO_4$–polyacrylamide gel electrophoresis	2×10^8	69

be bound to the immunoglobulins, or could the immunoglobulins themselves be partially removed from the Sepharose? Concerning the first possibility, it is unlikely that the antigen used for inoculation was impure (Figure 1). Secondly, it is unlikely that the immunosorbent column was acting entirely like a hydrophobic column because only a small amount of interferon (10%) was bound to a column prepared from control rabbit serum. To determine whether the impurities seen in Figure 3 were derived from proteins tightly bound to interferon, we additionally washed the immunosorbent column with the nonionic detergent Triton X-100 (0.1%) as well as with 50% ethylene glycol before elution with acetic acid. Neither wash removed much protein or interferon from the column. There was no major difference in the protein impurities of the interferon preparations eluted from such a column compared with the preparation in Figure 3, indicating that there are proteins tightly bound to interferon that are not readily dissociated by these treatments. To test for the possibility that some of these impurities (Figure 3) were derived from leakage of the proteins coupled to the Sepharose matrix, we eluted a control immunosorbent column not loaded with interferon with 100 mM acetic acid. Analysis of the eluant by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed the presence of noninterferon proteins similar to those in Figure 3. Thus, it is likely that some of the contaminants seen in Figure 3 were derived from the immunosorbent column itself. Attempts to eliminate this "leakage" of proteins from the column by partial purification of immunoglobulin as well as by different methods of coupling the immunoglobulin fraction to Sepharose have so far not led to much improvement. Nevertheless, the current immunosorbent column provides a quantitative procedure of purifying interferon after the first two preliminary steps. The potency of this method is illustrated by comparing Figure 3B, in which no interferon protein can be detected in CM-cellulose purified material, and Figure 3D, in which the interferon protein is clearly visible in the immunosorbent column purified preparation.

Alternatively, a two-step procedure is used to purify the fibroblastoid interferon when the interferon is produced by C-10 cell cultures in serum-free medium. Step one of this two-step procedure consists of purification and concentration of the crude interferon by immunosorbent column chromatography, and step two consists of purification by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis. The recoveries of interferon by these procedures are given in Table II. However, we do not normally produce C-10 interferon in serum-free medium because the amount of interferon produced in serum-free medium is 10–20% of the amount produced in the presence of fetal calf serum.

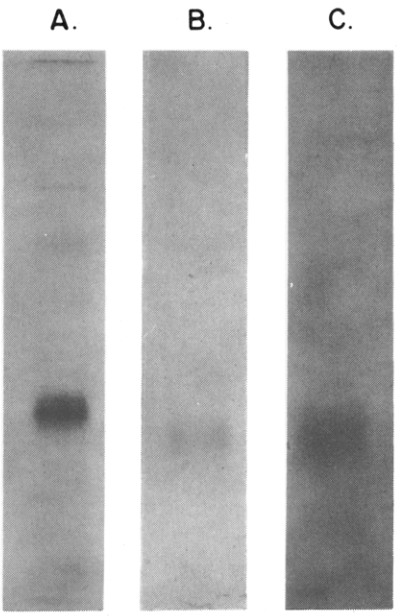

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of pure human fibroblastoid (C-10) interferon isolated by an improved procedure utilizing an immunosorbent column. (A) Protein labeled with ^{125}I using Bolton–Hunter reagent; (B) unlabeled, stained protein; (C) protein labeled with ^{125}I using the imido ester methyl hydroxybenzimidate.

Table III: Comparison of Amino Acid Compositions of Human Fibroblastoid Interferon Purified by Two Different Procedures

amino acid	by 2-D gel[a] (%)	by antibody column[b] (%)
aspartic acid and asparagine	12.1	11.1
threonine	5.2	5.5
serine	7.8	8.1
glutamic acid and glutamine	15.2	14.5
proline	2.5	2.2
glycine	3.2	3.5
alanine	5.4	5.1
valine	4.6	5.3
methionine	1.0	1.2
isoleucine	5.9	6.7
leucine	15.8	15.1
tyrosine	2.9	2.0
phenylalanine	4.5	5.8
histidine	2.7	1.4
lysine	7.2	8.4
tryptophan	0	ND[c]
arginine	5.01	4.1

[a] Tan et al. (1979). [b] As purified by the procedure described herein. [c] ND = not determined.

Purity and N-Terminal Amino Acid Sequence. The question of the purity of the interferon is now considered. Gel electrophoresis shows that the preparation contains a single molecular weight species of ~20000 (Figure 4). In all cases, interferon activity was found to comigrate with the stained protein fraction. When analyzed on acid–urea gel, the protein migrated as a discrete fraction with an R_f of 0.45, as previously reported for a pure human interferon preparation obtained by a different procedure (Tan et al., 1979). It was also possible to demonstrate the persistence of biological activity after the protein was stained.

Analysis of the amino acid composition (Table III) of the pure protein obtained by the immunosorbent procedure is

Met, Ser, Tyr, Asn, Leu, Leu, Gly, Phe, Leu, Gln, Arg, Ser, Ser[13]

FIGURE 5: N-Terminal amino acid sequence of C-10 interferon.

similar to that obtained by a previous procedure in which the purity of the interferon was described by several considerations (Tan et al., 1979). Direct confirmation of its purity is now obtained from the finding that there is only one N-terminal amino acid sequence for fibroblastoid interferon (Figure 5).

Though the initial N-terminal amino acid sequence of C-10 fibroblast interferon is similar to that of human diploid fibroblast interferon (Knight et al., 1980), the amino acid composition analysis of the two suggests that the C-10 interferon is slightly lower in its content of methionine (1%) and of tyrosine (2%) residues. At this time it is not known how these differences might be explained. Whether the difference is in peptide sequence will have to await the complete structural analysis of the two molecules in question.

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