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Purification of Mouse Interferon by Affinity Chromatography on a Solid-Phase Immunoadsorbent

(Newcastle disease virus)

JEAN D. SIPE*, JAQUELINE DE MAEYER-GUIGNARD†, BERNARD FAUCONNIER‡, AND EDWARD DE MAEYER†

* Electro-Nucleonics Laboratories, Inc., Bethesda, Maryland 20014; † Institut Du Radium, Faculté des Sciences, Bât. 110, Orsay 91405, France; and ‡ Faculte De Medicine, Rennes 35000, France

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ABSTRACT A solid-phase immunoadsorbent capable of binding mouse interferon has been prepared. Starting from crude tissue-culture material, interferon could be purified 1990 times in a single step of affinity chromatography. Overall recovery ranged from 55 to 103% with tissue culture and mouse-brain interferon; however, only 5% was obtained with Sendai virus-induced interferon from mouse serum.

Studies concerning the nature, and especially the mode of action, of the antiviral protein interferon consistently have been hampered by the lack of readily available, relatively pure material.

Previously described methods for purification of interferon have depended upon physicochemical properties of the molecule such as size, charge, and differential solubility (1, 2). Alternatively, affinity chromatography, which is a method of separation based on biological specificity or antigenicity, would seem particularly well suited for the purification of interferon, which is present in extremely low concentrations in biological materials (3). The technique, using antibodies covalently attached to cyanogen bromide-activated agarose (4, 5), has been used to purify several antigens (6).

We report here the preparation of a solid-phase immunoadsorbent for mouse interferon and describe its applicability for binding mouse interferon from several sources. These include the culture medium of induced mouse-embryo fibroblasts, mouse serum, and partially purified interferon from mouse brain.

MATERIALS AND METHODS

Interferons

Tissue Culture Interferon was prepared in secondary cultures of Swiss mouse-embryo fibroblasts by induction with Newcastle disease virus (NDV). The culture medium during induction was Eagle's minimal essential medium, enriched with 5% inactivated (30 min at 56°) calf serum. 24 hr after onset of induction, the culture fluids were harvested, acidified to pH 2.5, and left at 4° for 21 days. The fluids were then clarified by centrifugation at 4000 × *g* for 15 min and stored at −25° until use.

Mouse-Serum Interferon was induced through intravenous inoculation of C57BL/6 mice with Sendai virus; sera were obtained from the inoculated mice 12 hr later and stored at −20°.

Abbreviation: NDV, Newcastle disease virus; PBS, phosphate-buffered saline, pH 7.4.

Mouse-Brain Interferon was obtained through courtesy of I. Gresser. It had been extracted from IC mice inoculated with West Nile virus and partially purified by differential centrifugation and acidification (7).

Interferon assay

Interferon titers were determined by a plaque-reduction method in L cells, with vesicular stomatitis virus as challenge. One unit is defined as the minimal amount necessary to reduce the plaque number by 50%. One of our units corresponds to six international-reference units of mouse interferon.

Special care was taken to characterize the antiviral activity obtained after desorption, to ascertain that all activity measured was attributable to interferon. Species specificity was determined through complete lack of activity against vesicular stomatitis virus in chick-embryo fibroblast cultures and in two lines of simian cells, VERO and ES1. Furthermore, only between 5 and 10% of the activity observed in mouse L cells was found in rat-embryo fibroblast cultures; this ratio has been consistently found for mouse interferon. The antiviral activity was furthermore completely inactivated after incubation with trypsin (crystalline A grade, Calbiochem, Luzern, Switzerland) at a concentration of 100 μg/ml for 45 min at 37° and pH 7.6, whereas incubation with ribonuclease (pancreatic ribonuclease, crystalline A grade, Worthington Biochemical Co, Freehold, N.J., USA) at 10 μg/ml for 45 min at 37° had no effect. The antiviral activity was also completely resistant to dialysis against Sörensen buffer at pH 2 for 48 hr.

Chemicals

Bio-Gel A-15m, 50-100 mesh, was obtained from Calbiochem, Los Angeles, Calif., cyanogen bromide from Eastman Organic Chemicals, Rochester, N.Y., and urea from Schwarz/Mann, Orangeburg, N.Y. All were used without further purification.

Preparation of anti-interferon serum

The antiserum was obtained from a sheep that had been subcutaneously injected at weekly intervals for 35 weeks, with 40 ml of mouse L-cell interferon induced with NDV. The interferon preparation used for immunization was held at pH 2 for 5 days, brought to pH 7, and concentrated to 10^5 units/ml. The serum obtained was inactivated for 30 min at 56° before use. When diluted 800 times the serum was capable of neutralizing 10 units of mouse tissue-culture interferon.

TABLE 1. *Yield and specific activity of mouse interferon purified from various sources*

Exp.	Origin of interferon	Milliliters loaded	Specific activity of starting material (units/mg of protein)	Interferon, units absorbed to column	% Recovery	Specific activity	Purification (times)
1	Crude fibroblasts	2	2×10^2	1,000	76	6.5×10^3	31
2	Crude fibroblasts	240	2×10^2	84,000	55	4.1×10^5	1990
3	Brain	1	4.2×10^3	29,000	103	3.6×10^5	85
4	Serum	15	7	6,992	5	2.7×10^3	391

Preparation of anti-interferon and control immunoadsorbents

Control serum was obtained from an unimmunized sheep. The control serum and antiserum were each diluted with an equal volume of water and chilled in ice, then solid ammonium sulfate was added to give a final concentration of 2.1 M. After remaining at 4° for at least 16 hr, the precipitated immunoglobulins were pelleted by centrifugation at 10,000 × *g* for 10 min, and were washed twice with one-half the original volume of chilled 2.1 M ammonium sulfate.

The immunoglobulin fraction was dissolved in a minimum volume of 0.01 M phosphate buffer (pH 8.0) and dialyzed for at least 4 hr against 100 volumes of 0.01 M phosphate buffer (pH 8.0).

Procedures for washing and activation of Bio-Gel A-15m were those described by Cuatrecasas (5). The coupling reactions for control and anti-interferon immunoglobulins were performed in 0.01 M phosphate buffer (pH 8.0) with stirring at 4° for at least 30 min, followed by standing for at least 16 hr at 4°. The protein concentration was 15 mg/ml, and the settled bed volume of agarose was equal to the volume of protein solution. The amount of cyanogen bromide used for activation was 0.24 g/ml of settled bed volume of agarose. After coupling, the immunoglobulin–agarose was washed twice with 2 M urea and twice with 3 M NaCl; the volume of each wash was equal to the settled bed volume of agarose. Under these conditions, 80–90% of the added protein was linked. The immunoglobulin–agarose preparations were then equilibrated with phosphate-buffered saline (pH 7.4) (PBS), containing 100 μg/ml of penicillin and 50 μg/ml of streptomycin, and stored at 4° when not in use.

Operation of anti-interferon and control columns

The columns were equilibrated with PBS containing antibiotics before use. After the applied sample had fully entered the agarose, about 0.1 column volume of buffer was applied and allowed to enter. Then elution with PBS containing antibiotics was begun, at a flow rate of 0.2–0.3 ml/min. When the unabsorbed protein was rinsed from the column, desorption with 15 mM acetic acid containing 0.15 M NaCl was performed at a flow rate of 0.5–0.8 ml/min until the pH of the eluted fractions was 3.5. Except where specified, both sorption and desorption were performed at room temperature (24°). When not in use, a column was rinsed continuously with PBS containing antibiotics. For long-term storage of an affinity column, the immunoadsorbent was removed from the column and stored at 4° in PBS containing antibiotics.

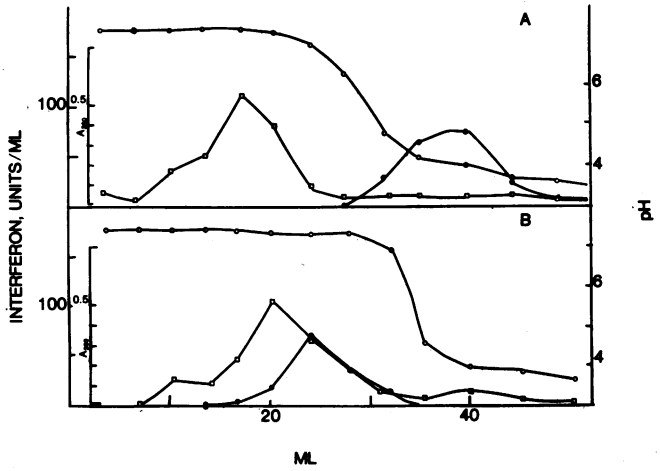

FIG. 1. Chromatography of crude fibroblast interferon on anti-interferon and control agarose columns. Whatman chromatographic columns (1.5 × 20 cm) containing about 20 ml of immunoglobulin-agarose were used. Anti-interferon–agarose (*A*) was prepared by coupling the immunoglobulin fraction from 10 ml of antiserum to 20 ml of agarose; control agarose (*B*) was similarly prepared from 10 ml of normal sheep serum and 20 ml of agarose. Operation of the columns and assay conditions are described in *Methods*. 880 Units were applied to each column. Recovery was 93% from column A and 69% from B. ●——●, interferon; □——□, A_{280}; ○——○, pH.

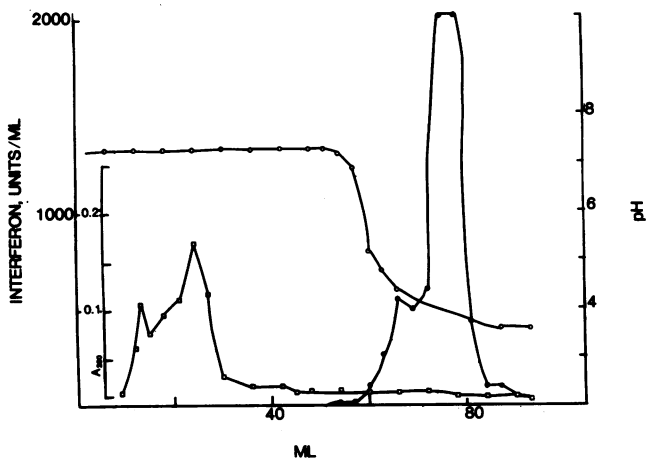

FIG. 2. Chromatography of mouse-brain interferon on anti-interferon–agarose. Conditions are the same as in Fig. 1, except that the volume of pH 7.4 eluant was approximately doubled. The figure refers to Exp. 3 of Table 1. Legends are the same as in Fig. 1.

Specific activity determinations

Protein was determined by the method of Lowry and coworkers (8). When necessary, fractions were concentrated by ultrafiltration through a Diaflo PM 10 membrane. (Amicon Corporation, Lexington, Mass., USA).

RESULTS

Binding and elution of unpurified mouse tissue-culture interferon

2-ml Aliquots of acidified crude interferon from mouse-embryo fibroblasts were neutralized and applied to the anti-interferon and control columns; one single-bed volume of PBS was then run through the gels. As shown by Fig. 1*A* and *B*, all of the interferon activity was washed through the control column at pH 7.4 while none of the interferon activity was found in the pH 7.4 fractions of the anti-interferon column. With the latter, interferon appeared with desorption buffer when the pH had dropped below 6, maximum activity being eluted at pH 4. Correspondingly, the interferon activity was eluted with the bulk of the sample protein on the control column, while on the anti-interferon column, it was resolved from most of the sample proteins.

When the volume of pH-7.4 wash was increased to about three bed-volumes, resolution of interferon from the other sample proteins was greatly improved.

In an effort to increase the specific activity of the isolated interferon, the sample size of crude interferon was increased from 2 ml to 240 ml. Sorption was performed at 4° and, after completely washing away unabsorbed protein, interferon was eluted at room temperature; the specific activity was increased about 2000 times, compared to that of the starting material, and 60 times, compared to that of the interferon obtained after desorption from the smaller sample (Table 1). Analysis of the pH-7.4 fractions from the larger sample showed that of the 96,000 units applied to the column, 84,000 were retained. This corresponds to the calculated capacity of the column, if we assume that there was no loss of antibody activity when the immunoglobulin fraction from 10 ml of antiserum (1 ml of which was capable of neutralizing 8000 units of interferon) was linked to agarose.

Binding and elution of mouse-brain interferon

When 1 ml of this preparation was chromatographed, the specific activity of the fraction of highest titer was increased 85 times (Fig. 2).

Binding and elution of mouse-serum interferon

15 ml of mouse serum containing 490 units/ml was applied to the column; however, only 5% of the input activity was desorbed at acid pH. Of the remaining 95%, only 5% was recovered with starting buffer. This is markedly different from the results obtained in the other preparations where the amount of interferon recovered in the pH-7.4 fractions was negligible when the capacity of the column was not exceeded. One possible explanation for the observation that only half of the recovered interferon was bound to the anti-interferon column is that mouse-serum interferon consists of molecules of different antigenicity. Indeed, *in vitro* neutralization tests indicate that, in contrast to fibroblast interferon, only a fraction of Sendai virus-induced serum interferon was neutralized by the antiserum used throughout this study. This phenomenon may be related to the observation of Duc-Goiran and coworkers (9), who reported an antigenic difference between human interferon made in white blood cells and in amnion cells. It should be recalled that Sendai virus-induced serum interferon is largely derived from lymphocytes (10). Another possible explanation for low recovery is that desorption of serum interferon may require a buffer of higher molarity than the one used throughout this study.

In spite of the low recovery, the serum interferon desorbed with 15 mM acetic acid–0.15 M NaCl was purified about 400 times and had a specific activity of 2.75×10^3 units of interferon per mg of protein.

DISCUSSION

The technique of affinity chromatography seems capable of yielding interferon preparations of high specific activity starting from crude material such as tissue culture fluid or serum. It should be stressed that the antiserum used for these experiments had been obtained by immunization with unpurified interferon, and therefore it also contained antibodies to calf-serum proteins and NDV and L-cell proteins, as well as to interferon. In the present experiments no attempt was made to remove these contaminating antibodies, and the degree of purification obtained with the tissue culture interferon is therefore quite remarkable, suggesting that much more purification will be obtained by use of more pure and potent antisera. The possibility that the antiviral activity obtained after desorption was not all attributable to interferon, but to a mixture of interferon and interferon-inducing material, was ruled out by characterization of the material as described in *Methods*.

A better antiserum could conceivably be obtained by immunization with interferon isolated by affinity chromatography from large quantities of mouse interferon synthesized *in vitro*. If another animal is then immunized with the culture medium from which interferon has been extracted, it will be possible to prepare a second immunosorbent with which to remove contaminating proteins from interferon isolated by affinity chromatography. Another way of reducing the amount of contaminating antigens isolated with interferon consists of starting with an interferon prepared in a system different from that used to make the interferon used for immunization. The potential of this approach is shown by the high degree of purification obtained after chromatography of mouse-brain interferon induced by West Nile virus; in this instance the anti-calf serum and anti-NDV antibodies on the column obviously do not interfere with obtaining a pure preparation. Particularly encouraging are the results obtained in the saturation study (Exp. 2). The degree of purification obtained with the saturated column was almost 2000 times, as compared to 31 times obtained when only 2 ml of the same tissue culture material was applied. This indicates that the amount of contaminating protein desorbed simultaneously with interferon reached its upper limit much sooner than did interferon, and would make the method especially suitable for simultaneous purification and concentration of large volumes. The fact that the same column can be used repeatedly is an additional asset.

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