Purified Rabbit Interferon: Attempts to Demonstrate Interferon-Specific ^3H -Protein

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^3H -protein present in highly purified preparations of rabbit interferon was not conclusively separated from noninterferon, cellular ^{14}C -proteins by co-chromatography on carboxymethyl-Sephadex or by co-electrophoresis on acid or neutral polyacrylamide gels.

The availability of isotopically labeled interferon (IF) would permit design of experiments to determine its cellular site of action as well as other hitherto elusive properties. Several laboratories have initiated such studies, which are essentially based on the empirical purification procedures described most extensively by Fantes and Furminger (1, 2). We wish to describe our attempts, similar to those of K. Paucker (*personal communication*), to identify labeled IF by several of these methods.

The system we chose for producing IF was primary cultures of rabbit kidney (RK) cells induced to synthesize IF by infection with the CG strain of Newcastle disease virus (NDV). As previously described (8), IF production in this system is rapid, antiviral activity is high, and crude preparations are of greater initial purity than many "purified" preparations. IF activity was titrated (8) by exposing triplicate monolayer cultures of 5-day-old RK cells to 1-ml dilutions of IF for 4 to 6 hr before challenge with 50 to 100 plaque-forming units (PFU) of vesicular stomatitis virus; the results were expressed as 50% plaque-depressing doses (PDD₅₀). Protein content of all fluids was measured by the method of Lowry et al. (3) by using crystalline bovine serum albumin as the standard. Proteins were labeled by incorporation of a mixture of ^3H -leucine (10.3 to 40.0 c/mmole) and ^3H -tyrosine (43.0 to 50.5 c/mmole) or uniformly labeled ^{14}C -amino acids (52 mc/matom). Radioactivity was determined by scintillation spectrometry of 50- μ liter samples or gel slices dissolved in *N*-chlorosuccinimide (NCS) and diluted in toluene-based fluors. In double-labeling experiments, simultaneous ^3H and ^{14}C disintegrations were calculated by the channels ratio method assisted by a computer program.

IF synthesis was induced in RK cells by infection with NDV at an input multiplicity of about 200 PFU. After adsorption for 30 min, the cell layers were washed three times and incubated at 37 C in special Basal Medium Eagle (BME) without serum and unlabeled leucine and tyrosine but containing ^3H -leucine and ^3H -tyrosine, each at a concentration of 10 $\mu\text{C}/\text{ml}$. Yields of IF at 8 hr were consistently 1,600 to 3,200 PDD₅₀/ml, and the specific activity of the crude IF ranged to 20,000 PDD₅₀/mg of protein. About a 10-fold purification was achieved by precipitation of inert cellular and viral protein with 0.15 N perchloric acid (PCA). The fluid was neutralized and the IF was concentrated by precipitation from the supernatant fluid made 0.02 M with respect to zinc acetate; the precipitate was resuspended in 0.2 N HCl and exhaustively dialyzed at 4 C against 0.1 M sodium phosphate, pH 6.0. Overall recovery of IF activity was 50 to 100% after these initial stages of purification and concentration. Further purification was achieved by chromatography on a column of carboxymethyl (CM)-Sephadex C50 equilibrated with 0.1 M sodium phosphate, pH 6.0. As described for other interferons (1, 5), rabbit IF was retained on the column at this pH; a considerable amount of inert protein and radioactivity could be removed by exhaustive washing at pH 6. IF was then eluted by means of a rising pH gradient of 0.1 M phosphate. Elution of IF activity consistently occurred as a peak at pH 6.4 to 6.6; these fractions contained IF purified as much as 1,000-fold with respect to the original protein concentration.

Table 1 shows representative results of purification of IF synthesized in the presence of ^3H -amino acids. Recovery of biological activity in pooled peak fractions was 12%, but specific activity increased 150-fold; most of the ^3H label (much of it undoubtedly amino acids as well as

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TABLE 1. *Purification of NDV-induced rabbit interferon*

Material	Vol (ml)	Total IF (PDD ₅₀)	IF activity (PDD ₅₀ /mg of protein)	Total ³ H (counts/min)
Culture fluid.....	400	640 × 10 ³	16 × 10 ³	1,900,000 × 10 ³
Perchloric acid supernatant fluid..	430	688 × 10 ³	160 × 10 ³	1,500,000 × 10 ³
Zinc precipitate.....	20	640 × 10 ³	188 × 10 ³	32,000 × 10 ³
CM-Sephadex peak fractions....	30	75 ^a × 10 ³	2,500 × 10 ³	8 × 10 ³

^a Twelve per cent of IF recovered in pool of three peak fractions. Total IF recovery from the column was 70%. In other experiments, the recovery of IF in pooled peak fractions was as high as 30%.

proteins) was removed. These results encouraged us to determine, by disc electrophoresis, whether the residual ³H label was associated with IF activity. For this purpose, we adopted the procedure described by Fantès (2) for electrophoresis of chick IF in acrylamide gels at pH 4.3. Some of these experiments were performed by preparative gel electrophoresis by using the Fractophorator (Buchler Instrument Co., Fort Lee, N.J.) which permits collection of fractions eluted into a reservoir at the gel front; recovery by this procedure of both IF activity and radioactivity was too low to be of practical value. Much better recovery of IF and ³H was obtained by elution from gel slices of polyacrylamide after electrophoresis in an analytical apparatus (Canalco, Rockville, Md.). Repeated runs of purified IF revealed coincident peaks of biological activity and ³H, but other peaks of radioactivity were distributed throughout the gel column. These results led us to perform what proved to be more definitive double-labeling experiments, in which ³H-proteins synthesized by NDV-induced cells and ¹⁴C-proteins synthesized by uninduced cells were mixed, purified concurrently, and examined simultaneously by disc electrophoresis. The elegant studies of Ptashne (6) served as a model for our experiments.

RK cells infected with NDV were incubated for 8 hr in special serum-free BME containing ³H-leucine and ³H-tyrosine, 10 μc/ml of each; uninfected control RK cells were incubated for 8 hr in BME containing ¹⁴C-amino acids, 1 μc/ml. In a representative experiment, 240 ml of media withdrawn from cultures of NDV-infected RK cells contained a total of 4.8 × 10⁵ PDD₅₀ of IF and 24 mg of protein. Media from uninfected RK cell cultures contained only 7.2 mg of protein in 360 ml. These media from IF-induced and uninduced cell cultures were mixed to give a specific activity of 16,000 PDD₅₀/mg of protein and purified sequentially by PCA and zinc acetate precipitation. About 9 mg of zinc-precipitated protein of specific IF activity ≅ 53,300 PDD₅₀/mg was chromatographed on a column of CM-Sephadex C50. IF activity began

to elute at pH 6.4; the peak was broad and considerable trailing occurred (Fig. 1). IF specific activity of pooled peak fractions was 1.6 × 10⁶ PDD₅₀/mg of protein, a 100-fold purification over the starting material. Much of the ³H and ¹⁴C radioactivity was eluted at pH 6.0, a finding which provides additional evidence of considerable purification. However, only relatively minor coincident peaks of ³H-protein were noted in the region of peak IF activity. In addition, there was no significant increase in the ³H to ¹⁴C ratios compared with that of prechromatographed IF.

Peak fractions of IF activity eluted from CM-Sephadex were pooled, concentrated by pressure dialysis or with Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), and analyzed by disc electrophoresis on columns of 7.5% polyacrylamide at pH 4.3 in β-alanine acetate buffer. Gels stained with Coomassie blue showed no visible protein bands. As shown in Fig. 2A, excellent correspondence was noted for the peak of IF activity with that of ³H radioactivity which migrated about 30 mm from the anode. Total recovery of IF from gel slices was almost 100%. A comparison of the ³H-protein concentration of the CM-Sephadex sample that was applied to the gel (see legend of Fig. 2A) with that which migrated to the region of peak IF activity (3,700 counts/min of ³H and 800 PDD₅₀ per slice of a gel run in parallel with the gel in Fig. 2A) revealed a further 60-fold purification by acid disc electrophoresis to give a specific IF activity of 12 × 10⁶ PDD₅₀/mg equivalent of ³H-protein. As a test of the purity of this highly purified interferon, peak fractions of IF activity and radioactivity eluted from two identical gels were subjected to re-electrophoresis under the same conditions. Figure 2B shows close correspondence of IF activity with a single ³H peak except for leading and trailing shoulders. Therefore, the ³H-protein associated with IF activity was relatively homogeneous. An additional fourfold purification was achieved by re-electrophoresis, giving a specific activity of 48 × 10⁶ PDD₅₀/mg equivalent of ³H-protein. In other

FIG. 1. Co-chromatography of a semipurified mixture of IF synthesized in RK cells in the presence of ^3H -amino acids and ^{14}C -proteins synthesized in control RK cells. A column of CM-Sephadex C50 (2.5 by 30 cm) was equilibrated with 0.1 M phosphate buffer (pH 6.0) and loaded with 30 ml of a sample containing 19.2×10^7 counts/min of ^3H , 3.2×10^7 counts/min of ^{14}C , 4.8×10^6 PDD₅₀ of IF, and 9 mg of protein. The loaded column was washed with 420 ml of buffer at pH 6.0, and a stepwise, rising pH gradient was established with 360 ml of 0.1 M phosphate buffer. The flow rate was 20 ml/hr, and fractions were 6 ml each. IF activity was measured by 50% plaque reduction by each appropriately diluted fraction. ^3H and ^{14}C radioactivity are expressed as percentages of total counts corrected for simultaneous counting of both isotopes.

words, one unit of this highly purified IF represented 2.0×10^{-11} g of protein.

Despite this evidence of high purity, no conclusion can be drawn concerning the actual incorporation of ^3H -leucine and ^3H -tyrosine into IF-specific protein. As noted in Fig. 2A, almost equivalent proportions of inert ^{14}C -protein peaked at exactly the same position in the gel. Although the ^{14}C counts were very low on re-electrophoresis (Fig. 2B), the ^3H to ^{14}C ratio in the peak IF fraction was similar to that in Fig. 2A.

One possible explanation for the above results is that IF could be a peptide chain complexed with an inactive carrier protein present in both induced and uninduced cells. Therefore, the method of Maizel (4) was used in an attempt to separate a possible IF peptide by treatment with acetic acid, urea, sodium dodecyl sulfate (SDS), and 2-mercaptoethanol, followed by electrophoresis on neutral urea-SDS-acrylamide gels as previously described (12). The biological activity of IF was completely destroyed under these conditions. Figure 3 reveals that none of the ^3H -peptides can be distinguished from non-interferon ^{14}C -peptides.

The molecular weight of IF purified by acid-disc electrophoresis was estimated by the method

FIG. 2. Comparative migration of purified RK IF and ^3H -proteins along with control cell ^{14}C -proteins co-electrophoresed in 7.5% polyacrylamide gels. (A) IF peak fractions from CM-Sephadex (Fig. 1) were pooled and concentrated by Ficoll; 0.2 ml of the concentrate contained 229,000 counts/min of ^3H , 34,000 counts/min of ^{14}C , 3,200 PDD₅₀ of IF, and 4 μg of protein. Samples of 0.2 ml each were loaded on gels and electrophoresed for 2 hr at 5 ma/gel in β -alanine acetate buffer, pH 4.5. The gel was first sliced lengthwise. Half of the gel was then sectioned into lengths of 1.25 mm; each slice was dissolved in hot NCS, and the ^3H and ^{14}C disintegrations were counted simultaneously. The other half of the gel was sectioned into lengths of 2.5 mm, and each slice was incubated for 24 hr at 4 C in phosphate-buffered saline (PBS; pH 7.2) containing 0.1% bovine plasma albumin; the eluate was assayed for IF by plaque reduction. (B) The pooled peak IF fractions from Fig. 2A and another identical acrylamide gel were concentrated to 4,200 counts/min of ^3H , 220 counts/min of ^{14}C , and 800 PDD₅₀ of IF per 0.2 ml. This concentrate was re-electrophoresed under the same conditions as in Fig. 2A. The whole gel was sliced transversely into 2.5-mm lengths and eluted in PBS; both isotopes and IF were measured in each fraction.

of Shapiro et al. (7) by electrophoresis on a neutral SDS-polyacrylamide gel. Although biological activity of IF is destroyed by SDS, a single peak of radioactivity should suggest homogeneity of the protein and permit estimation of its molecular weight. On this assumption, peak fractions of IF activity eluted from the gel shown

FIG. 3. Comparative electropherogram of ^3H -proteins synthesized by NDV-induced cells and ^{14}C -proteins synthesized by uninduced cells. A mixture of the two preparations was purified by differential precipitation and by chromatography on CM-Sephadex and then extracted with acetic acid, 0.5 M urea, 1% SDS, and 0.1% 2-mercaptoethanol, as previously described (12). A 200- μ l sample was loaded on a 7.5% polyacrylamide gel and electrophoresed for 5.5 hr at 5 ma in neutral phosphate buffer containing urea and SDS. The whole gel was sliced into lengths of 1.25 mm each for simultaneous counting of ^3H and ^{14}C . (See legend to Fig. 2.)

in Fig. 2A and four standard proteins (bovine serum albumin, ovalbumin, pepsin, and cytochrome c) were treated with 0.1% SDS and 2-mercaptoethanol and co-electrophoresed in a 5% acrylamide gel in the presence of SDS. The gel was stained with Coomassie blue and was scanned in a Gilford spectrophotometer at absorbancy at 620 nm with the aid of an automatic recorder to locate the stained protein peaks; radioactivity was counted in 1.25-mm sections. A single sharp ^3H -polypeptide peak migrated to a position in the gel equivalent to a molecular weight of 28,000 daltons. This compares with an estimated molecular weight of 45,000 for the biological activity of crude RK IF as determined by Sephadex filtration (9). These differences could be interpreted to mean that the ^3H -protein peak on the SDS gel was not interferon or that it represented dissociated chains of interferon.

These studies do not resolve the question of

whether interferons are synthesized de novo, which seems likely from other experiments (11), or whether they can exist in a precursor form that is activated by inducer. These data indicate that interferon, if it is indeed synthesized de novo in RK cells, cannot be readily distinguished from noninterferon proteins on the basis of charge or size. RK interferon may be derived partly from preexisting proteins, as was recently suggested by Vilček on the basis of his studies with protein inhibitors (10). There appears to be no method currently available for relating purified interferon to specific isotopically labeled protein.

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