Characterization of Interferon-associated Proteins

BY K. PAUCKER AND D. STANČEK*

Department of Microbiology, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129, U.S.A.

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SUMMARY

L cells in which interferon had been induced by u.v. irradiated Newcastle disease virus were exposed for varying periods of time to [3H]-L-methionine, [3H]- or [14C]-D-glucosamine, [3H]-protein hydrolysate or [14C]-L-fucose. Interferon was subsequently purified by SE-Sephadex chromatography and polyacrylamide gel electrophoresis to contain in excess of $1 \times 10^6$ units/mg. of protein. Electropho-grams disclosed that methionine, amino acid mixture and, to a lesser degree, glucosamine, were incorporated into proteins associated with the dominant interferon fraction. No incorporation of fucose was noted. Methionine and glucosamine labels were absent from the major interferon band in control preparations from non-induced cells, co-electrophoresed in acrylamide gel with non-labelled chromatographed marker interferon.

INTRODUCTION

Earlier reports described the approximately 1000-fold purification of L cell interferon by a sequence of procedures which involved acidification, chromatography and polyacrylamide gel electrophoresis (Paucker et al. 1970; Stanček & Paucker, 1970b). In spite of the high specific activity of such materials (up to $1 \times 10^7$ units/mg. protein), interferon appeared heterogeneous both on electrophoresis in polyacrylamide gel and isoelectric focusing (Stanček, Gressnerová & Paucker, 1970c). Residual proteins labelled with radioactive amino acids after induction of L cells by u.v. irradiated Newcastle disease virus remained associated with interferon activity. The nature of these proteins is obscure and it is not certain whether they represent in whole or in part interferon, contaminating normal host constituents or virus-induced proteins. They have been designated under the descriptive term ‘interferon-associated’ which leaves the basic question of their origin open to further inquiry.

Previous studies on the physicochemical properties of interferons were recently reviewed by Fantes (1970). Among the chemical constituents that may be important for interferon activity, carbohydrates and methionine were prominently mentioned. In particular, preliminary analysis of purified chick interferon disclosed a relatively high content of glucosamine (quoted in Fantes, 1966), and fucose (along with galactose and glucose) was implicated in the composition of purified rabbit inhibitory factor (Nagano et al. 1965). Both chick and rabbit interferons were said to require one or more methionine residues for expression of antiviral function (Fantes & O'Neill, 1964; Ke & Ho, 1968). Accordingly, radioactive L-methionine, D-glucosamine and L-fucose were selected for labelling experiments to see whether any of these precursors would be incorporated into those proteins which on the basis of charge and size could not be separated from highly purified mouse interferon.

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METHODS

Cells. L cells were grown in suspensions as previously described (Paucker, Skurska & Henle, 1962), except for the use of Eagle basal medium in spinner salt solution, supplemented with 10% calf serum and antibiotics. For attachment to glass, Eagle basal medium (Hanks base, HBME) containing 10% calf serum, 0.15% sodium bicarbonate and antibiotics, was substituted.

Interferon was assayed in monolayers of L (MCN) cells (Rothfels et al. 1959), propagated in Scherer’s maintenance solution, 60%; medium 199, 30%; and 10% inactivated (56°, 30 min.) horse serum.

Viruses. The VICTORIA strain of Newcastle disease virus (NDV) passaged in chick embryos was employed for stimulation of interferon. The chick embryo-adapted Indiana type of vesicular stomatitis virus (VSV), carried subsequently through 40 passages in high dilutions in L cells, was used for challenge in interferon titrations.

Inducers. For production of interferon, NDV-containing allantoic fluids were subjected to dialysis against 0.01 M-phosphate buffered saline (PBS) at pH 7.2 and subsequently to u.v. light, as reported earlier (Paucker et al. 1970). The irradiated virus is referred to as NDVuv.

Production and labelling of interferon. The detailed procedure was described elsewhere (Paucker et al. 1970). In essence, round 500 ml. prescription bottles containing confluent monolayers of from 5 to 10 x 10⁷ cells were washed twice with warm Hanks solution prior to stimulation by NDVuv at an input multiplicity of 300 EID 50 equivalents/cell for 1 hr. The inducer was then removed, the cells were washed again and the cultures were re-incubated in fresh medium, but in the absence of serum.

Isotopic label was added to the medium of non-induced and induced cultures at the times indicated in the text. The following isotopes were employed: [3H]-protein hydrolysate (Schwarz/Mann, Orangeburg, N.Y., Cat. No. 3130-08); [14C]-l-fucose, specific activity 1 to 2 mc/m-mole (International Chemical and Nuclear Corporation, Irvine, California, Cat. No. 11148); [3H]-methyl-l-methionine, specific activity 185 mc/m-mole; [3H]-d-glucosamine, specific activity 1300 mc/m-mole, and [14C]-d-glucosamine, specific activity 107 mc/m-mole (New England Nuclear, Boston, Mass., Cat. Nos. NET-061, NET-190, NEC-193, respectively). Isotopes were made up in serum-free growth medium at the concentrations indicated. In the case of methionine and protein hydrolysate, amino acids normally present were also eliminated from the medium. After removal of labelled medium and prior to re-feeding at any step in the procedure, the monolayers were extensively washed with the Hanks solution.

Media containing interferon and corresponding control materials were harvested during the time intervals mentioned in the text. The preparations were then concentrated about 50-fold by ultrafiltration, acidified to pH 4.5 (Schonne, 1966) to precipitate and remove inert protein and exhaustively dialysed against 0.1 m-phosphate buffer at pH 6.0 to remove low molecular weight label.

Chromatography. Interferon and control materials were subjected to ion exchange chromatography in the cold as described (Paucker et al. 1970), except that sulfoethyl (SE) Sephadex C-25 was substituted for carboxymethyl Sephadex C-25. Preparations were adsorbed at pH 6 and elution proceeded in a rising pH gradient at constant molarity of the buffer. Interferon eluted in a broad zone ranging from pH 6.8 to 10.5. The corresponding fractions were pooled and crystalline bovine plasma albumin (BPA) was added to give a final concentration of 0.5% (w/v) after re-concentration of the pools by ultra-filtration.

Polyacrylamide gel electrophoresis. Concentrated chromatographed eluate was subjected
to electrophoresis at pH 4.3 in a Canalco Model 12 electrophoretic apparatus as reported elsewhere (Staněck & Paucker, 1970a; Paucker et al. 1970), except that longer gel columns (160 to 200 × 5 mm.) and a correspondingly longer migration time were usually employed. The presence of BPA during electrophoresis aided the recovery of biological activity but did not otherwise interfere with the distribution of interferon in the gels (Staněck & Paucker, 1970b).

Interferon was eluted in the cold for several days, usually from pools of two 1 mm. discs, into 0.5 ml. volumes of PBS containing 0.5 % BPA (w/v). For measuring radioactivity, samples of 0.1 ml. were dispensed into vials containing Ready-Solv Solution VI (Beckman Instruments, Fullerton, California) and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Illinois). Purification was assessed in terms of reduction of radioactive counts/min./unit of interferon, since the presence of BPA precluded direct protein measurements.

Proteins. To determine the specific activities of the initial crude interferons, protein content was measured by the method of Lowry et al. (1951), using BPA as a standard.

Interferon assay. Interferon was titrated on monolayers of L (MCN) cells by measuring the reduction in the number of plaques of VSV (Paucker et al. 1970). Both internal and NIH reference standards accompanied each titration. Based on a total of 18 independent titrations, one unit of interferon (IFU) as defined in this assay corresponded to 32 NIH mouse interferon reference units.

RESULTS

Incorporation of [3H]-methionine and [3H]-protein hydrolysate into interferon-associated proteins

Three groups of L cell monolayer cultures consisting of 13 roller bottles each were handled as follows: two sets were induced by NDVuv as referred to under Methods and the third group was not treated at this time. One hour later all monolayers were re-fed with serum-free medium of reduced (1/20) amino acid content, and re-incubated for an additional 7 hr. Groups 1 and 2 which had been induced by NDVuv were then given serum-free medium containing either [3H]-protein hydrolysate (17 μc/ml.) or [3H]-L-methionine (17 μc/ml.), respectively, and the third group of non-induced cells received also [3H]-L-methionine at the same concentration. All cultures were then returned to the incubator for an additional 4 hr at the end of which the media were collected and further processed as described under Methods. Input preparations for chromatography were adjusted to contain 1 mg. of protein/ml., which necessitated an approximately 30-fold concentration for interferon and a 100-fold concentration for fluids in the control group. For electrophoresis in polyacrylamide gels, a non-labelled chromatographed NDVuv-induced L cell interferon was admixed to the material from non-induced cells (group 3) to help locate the position of interferon in relation to radioactivity. Electropherograms of these preparations are shown in Fig. 1.

Interferon activity in all three experimental groups revealed a similar distribution. A sharply delineated, rapidly migrating band was followed by a more slowly moving, diffuse interferon zone. In the material derived from non-induced cultures, there were no labelled proteins which corresponded to the distribution of the admixed marker interferon in the gel. On the other hand, [3H]-proteins were found to be distinctly associated with the sharp interferon bands from NDVuv-induced cells which had been subsequently labelled with either methionine or protein hydrolysate. However, isotope distribution was non-specific in the trailing areas of interferon activity in both groups. In addition, the radioactive migration profile of the preparation from cultures which had received amino acid mixture, included
proteins in an essentially interferon-free region. These were largely absent in the methionine-labelled group.

Lack of $[^{14}C]$-fucose incorporation into interferon-associated proteins

Twenty monolayer cultures of L cells in round prescription bottles were first exposed to NDVuv and then re-incubated in serum-free medium containing 0.5 μc/ml of $[^{14}C]$-fucose. Incubation proceeded for a total of 16 hr to allow for maximal incorporation of the relatively small amount of isotope added. The harvested interferon-containing culture fluids were concentrated, acidified and chromatographed in the usual manner. The approximately 200-fold purified material was subsequently subjected to polyacrylamide gel electrophoresis.

As shown in Fig. 2 (see legend for details), interferon activity was spread throughout the middle portion of the gel. Radioactivity remained at background level and there was no evidence of labelling of any of the interferon components. However, the low radioactivity in the input material may have precluded more exact determinations.

Incorporation of $[^{3}H]$-glucosamine into interferon-associated proteins

Twenty L cell monolayer cultures in round prescription bottles were induced with NDVuv, and re-fed with serum-free medium containing 5 μc of $[^{3}H]$-glucosamine/ml. Interferon was collected 16 hr later and subjected to the usual purification steps. A parallel group of non-induced cells was labelled and handled in the identical manner. As a last step, both groups were subjected to electrophoresis in polyacrylamide gels. The corresponding profiles are shown in Fig. 3.

In the case of the non-induced group, the tritium label was distributed throughout most
Fig. 2. Electropherogram of chromatographed L cell interferon from cultures labelled with [\(^{14}\)C]-\(\text{L-fucose}\) after induction by NDVuv. Gel was loaded with 0.4 ml containing 121,60 IFU and 300 counts/min. Recovery of interferon (○—○) was 53 % and of isotope (●—●) 130 %.

Fig. 3. Electropherogram of chromatographed NDVuv-induced L cell interferon and control proteins labelled with [\(^{3}\)H]-\(\text{D-glucosamine}\). (a) Non-induced cells: 7-5 % gel, 2 mA/gel for 15 min., then 5 mA/gel for 8½ hr; two 1 mm. discs/fraction. Input: 5250 counts/min. (●—●) admixed with 400 IFU of non-labelled chromatographed interferon as a marker (○—○). (b) NDVuv-induced cells: 9 % gel, 5 mA/gel for 5½ hr; two 1 mm. discs/fraction from no. 1 to 30, single disc/fraction for remainder of gel. Input: 33,063 counts/min., 4800 IFU. Interferon activity in fractions, ○—○; radioactivity in fractions, ●—●. Recoveries: 3833 counts/min. (11.6 %), 1026 IFU (21.4 %).

of the gel and with the exception of a minor peak in fraction 56, none of it coincided with the major area of activity in the co-electrophoresed non-labelled chromatographed interferon added as a marker. NDVuv-induced interferon was broadly distributed over an area covering some twenty fractions, but nearly half of the recoverable activity was confined to only five fractions. Approximately 70 % of the eluted isotope migrated more slowly than interferon with two major peaks in essentially interferon-free fractions 18 and 20. These might represent virus-induced products since no comparable profile is seen with materials derived from
control cells. However, more than two-thirds of the remaining tritium counts were clearly coincident with the dominant interferon band.

In comparing the migration profiles shown in Fig. 3, it can be seen that the major interferon components do not occupy similar positions in the two gels. This discrepancy is due to the fact that the preparations were not electrophoresed concurrently and somewhat different conditions were employed, as detailed in the legend.

A prolonged interval for labelling with $[^{3}H]$-glucosamine had been used in the preceding experiment to permit maximum incorporation of the isotope. To allow a better comparison with earlier studies in which shorter labelling periods were used, and to minimize reconversion of already incorporated isotope, the following experiment was carried out.

Fifty monolayers of L cells in roller bottles were induced by NDVuv and incubated in serum-free medium containing $1/20$ of the usual amount of amino acids for an additional 9 hr. The cultures were then re-fed with serum- and amino acid-free medium containing 2 $\mu$C/ml. of $[^{14}C]$-d-glucosamine and 20 $\mu$C/ml. of $[^{3}H]$-protein hydrolysate, and returned to the incubator for an additional 5 hr. Interferon was collected 16 hr following initial exposure to NDVuv and partially purified by the usual procedures including SE-Sephadex chromatography. The concentrated chromatographic eluates were then subjected to polyacrylamide gel electrophoresis as shown in Fig. 4. Interferon activity was essentially confined to two portions of the gel, i.e. a more slowly migrating non-labelled broad zone extending from fractions 25 to 50 and a sharply defined, more rapidly moving area between fractions 57 and 61 with which label was associated. The two portions account for 52 and 19%, respectively, of the total recoverable interferon titre. The $[^{3}H]$-proteins which coincided with the interferon band represented 24% of the total tritium elutable from the gel. Furthermore, $[^{14}C]$-activity was distinctly associated with peak fraction 58 and accounted for approximately 10% of

![Graphical representation of the electropherogram showing interferon activity and label distribution.](image-url)
Fig. 5. Re-electrophoresis of fraction no. 58, Fig. 4, of chromatographed L cell interferon, double-labelled with [3H]-protein hydrolysate and [14C]-d-glucosamine after stimulation by NDVuv. Input: 4800 [3H]-counts/min., 104 [14C]-counts/min., 420 IFU. Recoveries: 4210 [3H]-counts/min. (88%), [14C]-counts/min. not determined, 400 IFU (95%). Interferon activity in fractions, ○—○; radioactivity in fractions, •—•.

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DISCUSSION

The addition of labelled amino acid mixture to L cells, induced by NDVuv, has permitted the identification of proteins which were not only synthesized simultaneously with interferon, but which corresponded at least in size and charge to the major interferon component in acrylamide gels (Paucker et al. 1970). What is more, such proteins were not liberated spontaneously from non-stimulated cells in quantities sufficient to be similarly analysed (Stanček,
The heterogeneous composition with respect to microelectric charge of L cell interferon (Staněk et al. 1970c) which is shared by chick (Fantes, 1969) and rabbit interferons (Schonne, Billiau & De Somer, 1969) is likely to account in part for the fairly broad distribution of interferon activity particularly evident in longer gels (see Fig. 4). The amino acid label is not strictly associated with the more slowly moving (possibly larger) interferon proteins which suggests that they may have been synthesized prior to the addition of isotope. It is improbable, therefore, that they represent aggregates of monomeric forms (Carter, 1970, 1971). Analysis of results has thus been confined to the dominant interferon band where juxtaposition of label and interferon has been well established.

In the absence of a pure product it is difficult to study the physicochemical characteristics of interferons (Fantes, 1970). However, valuable preliminary information has been gained by the judicious use of chemicals known to affect more or less distinct molecular configurations. The most specific of these appears to be cyanogen bromide which selectively reacts with methionine under defined conditions and has been applied to the non-enzymatic cleavage of the methionyl peptide bond (Gross & Witkop, 1960). This reagent was also shown to inactivate both purified chick (Fantes & O'Neill, 1964) and crude rabbit interferons (Ke & Ho, 1968). Although comparable inactivation studies were not carried out with L cell interferon, basic similarities common to all interferons suggested that methionine may also be required for the antiviral function of mouse interferon.

Labelling of L cells with [3H]-methionine after induction by NDVuv disclosed that some of the precursor was indeed incorporated into proteins which corresponded precisely to the fastest and most homogeneous interferon component in the electrophoretic migration profile. Incorporation of methionine was more selective than that obtained with protein hydrolysate, since the isotope was more specifically associated with interferon activity and less so with other more slowly migrating proteins. The significance of the latter is not clear, but their absence in controls suggests that they may represent other virus-induced components. It would thus seem that the use of a single labelled amino acid of high specific activity and more selective association with interferon than the amino acid mixture previously employed, would warrant consideration in future studies on the nature of interferon-associated proteins.

The participation of carbohydrates in the composition of interferons was first suggested by Nagano, Kojima & Suzuki (1960), who found that the antiviral activity of rabbit inhibitory factor was abolished by treatment with periodic acid. The same group proposed the attractive hypothesis that the active antiviral site of the inhibitory factor consisted of a protein-free oligo- or polysaccharide which is loosely bound to a variety of inactive proteins (Nagano et al. 1965). However, since the oxidizing action of periodate is not limited to carbohydrates (Clamp & Hough, 1965; Dixon, 1962; Lee & Montgomery, 1961), the significance of such data is not clear. More direct methods to detect polysaccharides in highly purified chick interferon indicated that trace amounts of sugars may be present (Lampson et al. 1963; Fantes & Furminger, 1967), but the participation of possible impurities could not be ruled out (Fantes & Furminger, 1967). Treatment with sialidase does not appear to affect the biological activity of rabbit interferon (Viti et al. 1970) although such exposure may alter its isoelectric characteristics (Schonne et al. 1969).

The data presented here indicate that glucosamine is incorporated to a minor but nevertheless distinct degree into interferon-associated proteins. While the smaller amount of isotope used in these experiments may account to some extent for the lesser incorporation achieved with glucosamine than with methionine or protein hydrolysate, it is probable that polysaccharides are less prominently represented in these proteins. Nevertheless, the possi-
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bility remains that some of the added glucosamine label underwent subsequent conversion to other metabolic products, notably amino acids. However, the finding that more prolonged incubation with the isotope did not promote enhanced incorporation suggests that no major re-utilization of glucosamine had occurred. Lack of incorporation of fucose, which is metabolically very stable, could have been due to the small quantity of isotope used but more likely reflects the more selective distribution of this carbohydrate among naturally occurring proteins.

Neither methionine nor glucosamine labels appear to peak with interferon that had been admixed with labelled control materials from non-induced cells prior to acrylamide gel electrophoresis. This may be explained by the reduced liberation of proteins from non-stimulated cultures (Staněck, Golgher & Paucker, 1970b) or their selective elimination during various purification steps (Paucker et al. 1970). More extensively concentrated preparations derived from non-induced mouse (Staněck et al. 1970a) and rabbit cells (Yamazaki & Wagner, 1970) are known to contain proteins which cannot be separated from purified interferon on the basis of size and charge.

In essence, the results indicate that the activity of extensively purified L cell interferon remains associated with proteins formed after induction, which could be labelled with methionine and to a lesser extent with glucosamine. While these and earlier data by other investigators support the concept of the glycoprotein nature of interferon-associated proteins, if not of interferon itself, final verification of their identity may have to be provided by immunologic approaches. Such studies are now in progress.

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