Apparent Dispensability of the Carbohydrate Moiety of Human Interferon for Antiviral Activity

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Human leukocyte and tritium-labeled fibroblast interferons, prepared by induction with Sendai virus and with double-stranded polyinosinic acid-polycytidylic acid respectively, have been studied in relation to the carbohydrate moieties attached to them. These interferons were partially purified by immunosorbance and by gel filtration. On treatment with glycosidases about 80% of the I-labeled sugar moieties in this glycoprotein-containing fraction was removed without detectable alteration of the antiviral activity or antibody-binding properties characteristic of interferon. The molecular weight of leukocyte interferon was reduced by about 4000. As others have reported, the heterogeneous character of interferon revealed by isoelectric focusing was greatly reduced by the enzyme treatment.

The study of the purification and properties of interferon has attracted much attention because of the potentially valuable antiviral activity of this substance. It has not yet been possible to produce interferon in large quantities by tissue culture. Consequently, many investigators have focused either on its isolation in pure form, for the purpose of determination of structure and, possibly, for future chemical synthesis, or on the development of potent, nontoxic inducers to stimulate endogenous interferon production.

Schonne et al. (1) observed a decreased charge heterogeneity of rabbit interferon upon isoelectric focusing following treatment with neuraminidase. They proposed that interferon is a glycoprotein, possessing variable amounts of sialic acid. Later, this hypothesis was confirmed by Dorner et al. (2). Rabbit interferon, upon treatment with neuraminidase, exhibited a single peak focusing at pH 6.3. This asialo-interferon regained its original heterogeneous character after reincorporation of sialic acid residues by the use of sialyltransferase. Also, the terminal sequence of the carbohydrate moiety was identified as sialic acid → galactose.

The studies of Schonne et al. (1) and of Dorner et al. (2), referred to above, have shown that terminal sialic residues may be removed without loss of antiviral activity. In the case of the latter studies (2), the next carbohydrate residue in the polysaccharide chain, galactose, could be successively oxidized with galactose oxidase and reduced with sodium borohydride without loss of activity. Since most glycoproteins studied to date contain one or more polysaccharide chains with molecular weights of 1000 to 3000, the present studies were carried out to examine the effects of more extensive carbohydrate removal.

Such studies are of importance in connection with the feasibility of eventual synthesis. This paper summarizes experiments on human leukocyte and fibroblast interferon that indicate the dispensability of the bulk of the carbohydrate component.

MATERIALS AND METHODS

Human leukocyte interferon prepared from human leukocytes induced with Sendai virus by H. Strander and K. Cantell (3) and Smith, Kline and French Laboratories was supplied to us by the Antiviral Substances Program of the National Institute for Allergy and Infectious Diseases. Fibroblast interferon was prepared with the kind cooperation of J. Vilcek and E. Hasel at New York University, using the FS-4 strain of human foreskin diploid fibroblasts induced with a double-stranded complex of polyinosinic acid-polyribidylic acid (4). Tritiated glycosides were incorporated into interferon preparations by adding n-[3H]glucosamine-hydrochloride with a specific activity of 10.13 Ci/mM (New England Nuclear, 3 mCi/100 ml of culture fluid) to Eagle's minimal essential medium with a glucose concentration lowered to 20 mg/100 ml. The tissue culture fluids were then desalted by passing them through a column of Sephadex G-25 medium (0.2 × 75 cm) in 0.1 M acetic acid to remove the bulk of the radioactivity.

The I-labeled protein fraction from fibroblast cultures was purified by immunosorbance chromatography (5, 6) and further by gel filtration on Sephadex G-100 (fine 2.6 × 90 cm). Human leukocyte interferon was subjected to the same purification procedures. Interferons were eluted from the affinity column by a pH gradient (6) containing cytochrome c (0.3 mg/ml) and from G-100 columns by 0.1 M acetic acid containing NaCl (0.15 M) and cytochrome c (0.5 mg/ml). The partially purified interferons were dialyzed against 0.1 M acetic acid and lyophilized.

Interferon assays were carried out by a modification of the micro method originally described for rabbit kidney cells (7). The reference human interferon preparation, 99/18, (titer 3 × 10^9 units/ml) in this assay. The specific activities of partially purified interferons have not been estimated in the current studies. As discussed earlier (6), a large purification (200 to 300-fold) is obtained in the first step (immunosorbance on antibody-Sepharose columns). Indeed, the amounts of protein present in the interferon fraction is sufficient such that it is necessary to add a protective protein (in our experiments, cytochrome c) to prevent losses due to adsorption in subsequent steps. The current studies are concerned with the physical behavior before and after removal of carbohydrates. The changes in charge and mass have been

related to the migration of material possessing antiviral activity during
isoelectric focusing and gel filtration.

The mixture of glycosidases used to partially remove the carbohydrate moiety from interferon was prepared from Diplococcus pneumoniae, Type 1, with the kind cooperation of G. Ashwell, National Institute of Arthritis, Metabolism, and Digestive Diseases. α-Mannosidase (49 units/ml) was a gift from Dr. Ashwell. The glycosidase preparation (8, 9) contained β-galactosidase (4.6 units/ml), N-acetylglucosaminidase (10.4 units/ml), neuraminidase (0.1 units/ml) and an undetermined amount of endoglycosidase (10).

The protein concentration of this preparation was 3.9 mg/ml.

Treatment of 4H-labeled Interferon with Glycosidases—The partially purified and lyophilized interferon was redisolved in Methylamine phosphate/citrate buffer, pH 6.0, and the initial radioactivity was determined. Solutions of glycosidases (2 μl/1000 units of interferon) and α-mannosidase (0.25 μl/1000 units of interferon) were added and the mixture was incubated at 37°C. Aliquots (0.2 ml) were taken during the course of the enzymic reaction for interferon assay. For the determination of sugar released, aliquots (0.1 ml) were removed and precipitated by addition of 0.2 ml of 2% phosphotungstic acid in 0.5 N HCl. After centrifugation, a measured amount of the supernatant was counted in a liquid scintillation counter.

For experiments designed to identify the 4H-labeled sugars removed by enzyme treatment, the interferon was dissolved in deionized water rather than buffer. After 3 hours incubation with glycosidases alone (2 μl/1000 units of interferon), the reaction mixture was passed through a column (1.6 x 28 cm) of Sephadex G-25 medium equilibrated with 0.1 M acetic acid. The fractions containing the low molecular weight material were lyophilized and redissolved in 0.5 ml of water. A 0.1-ml aliquot was applied together with a carrier mixture of mannose, galactose, sialic acid, and N-acetylglucosamine (1 μg each) on a Whatman No. 1MM paper. The same mixture was also applied for reference and the paper was subjected to descending chromatography using as solvent system, pyridine/acetic acid/ethyl acetate/water (6/3/5/3, v/v). The reference strip was developed with silver nitrate-staining solution (11) and the corresponding areas of the strip with the radioactive sample were cut out and counted in a liquid scintillation counter.

Antibody-binding Experiments—Antibodies to human leukocyte interferon, prepared in sheep, were purified and bound to Sepharose as described previously (6). Leukocyte and fibroblast interferons, after treatment with enzymes, were passed through a column (1 x 5 cm) prepared from the Sepharose-bound antibodies. The column was washed with phosphate-buffered saline and eluted at pH 2.2 with Sorensen’s citrate buffer containing cytochrome c (0.5 mg/ml).

Isoelectric Focusing—Leukocyte interferon (6.7 x 109 units) before and after treatment with glycosidases and α-mannosidase for 3 hours as mentioned above for fibroblast material, was subjected to isoelectric focusing at 4°C using pH 3.5 to 8.0 Ampholines (LKB, Bromma, Sweden) and a 25-ml sucrose gradient of 0 to 50% (Fig. 1). After 5 hours at 310 volts, 0.5-ml fractions were collected and their pH was measured. Fractions were assayed for interferon activity.

Molecular Weight Determination—The apparent molecular weight of human leukocyte interferon was estimated by gel filtration using a Sephadex G-100 (fine) column (1.5 x 100 cm) equilibrated with 0.1 M acetic acid containing 0.15 M NaCl. Ovalbumin, chymotrypsinogen, and bovine pancreatic ribonuclease were added as markers and dextran blue was used to determine the void volume (elution followed at 280 nm on a LKB Uvicord). The flow rate was maintained at 10 ml/hour and 1-ml fractions were collected. The eluates were examined at 280 nm for protein concentration, and assayed for interferon and ribonuclease (12) activities. The determination was carried out on leukocyte interferon (109 units) both before and after treatment with glycosidases and α-mannosidase.

RESULTS AND DISCUSSION
As can be seen in Table I, incubation of the protein fraction containing fibroblast interferon labeled with tritiated sugar moieties with a mixture of glycosidases and α-mannosidase resulted in the progressive removal of tritiated sugar residues. Between 74 to 85% of the tritium label was removed without loss of interferon activity in three experiments carried out on different preparations of tritiated interferon. Complete re-

FIG. 1. Isoelectric focusing of human interferon before (a) and after (b) removal of the carbohydrate moiety by treatment with glycosidases for 3 hours as described under "Materials and Methods." Human interferon (leukocyte, 6.7 x 109 units in each case) was focused at pH 3.5 to 8 in a 0 to 50% sucrose gradient. •, pH profile; O-O, interferon activity.

moval of the tritiated sugars was not achieved, even with longer enzyme incubation times. This could be due to the fact that part of the N-acetylglucosamine residues are directly connected to the polypeptide chain of the glycoprotein and that the sugar-amino acid bond cannot be enzymatically cleaved. However, the fact that the molecular weight (Fig. 2) of leukocyte interferon is considerably decreased by glycosidase treatment indicates that a large part of the sugar attached to interferon has been removed.

The tritiated sugars cleaved from the interferon-containing glycoprotein fraction by treatment with glycosidases alone were separated by paper chromatography. Of the total radioactivity applied to the paper (572 dpm), 124 dpm were recovered in the spot corresponding to sialic acid (Rf = 0.50) and 348 dpm as N-acetylglucosamine (Rf = 0.68). No significant radioactivity was detected in any other part of the paper strip. Thus, some conversion of the glucosamine, added to the culture medium during interferon production, to sialic acid by condensation with phosphoenolpyruvate and to N-acetylglucosamine by reaction with acetyl-CoA had taken place.

Concentration of glucosamine to other sugars commonly occurring in glycoproteins would not be expected under our experimental conditions.

Treatment of fibroblast interferon, prepared in the presence of tritiated glucosamine, with neuraminidase alone resulted in the removal of 25 to 30% of the tritium label and in this case the only product detected was [3H]sialic acid. This agrees with the findings of Dorner et al. (2) for rabbit interferon, namely
that sialic acid is present as the terminal residue in the carbohydrate moiety of this material.

Neither leukocyte nor fibroblast interferons showed any change in binding to the anti-interferon antibody column as a result of treatment with glycosidases and a-mannosidase (Table II) under the conditions described under "Materials and Methods." The A

\[ M \] of the marker proteins of the three columns were completely indistinguishable when they were superimposed; 1-ml fractions were collected. --- interferon activity of (O) untreated and (O) enzyme-treated interferon. The experimentally determined values for interferon activity were fitted by Gaussian curves, assuming each point to have the same statistical value.

This indicates that the removal of much of the carbohydrate from interferon does not affect its ability to bind to its specific antibodies.

Isoelectric focusing experiments on both types of interferons indicate that after partial removal of the carbohydrate moiety from interferon, its heterogeneous character is reduced (Fig. 1). The recovery of leukocyte interferon (6.7 x 10^3 units) on isoelectric focusing was 36%, whereas the recovery after enzymic removal of the carbohydrate moiety was 71%. This type of heterogeneous behavior on isoelectric focusing was previously observed by Schonne et al. (1) using rabbit interferon and by Stanček et al. (13) using rabbit, mouse, and human interferon. The heterogeneity was reduced after treatment with neuraminidase (1, 2). Dorner et al. (2) showed that the terminal sugar residue of the carbohydrate moiety was sialic acid.

The results of molecular weight determinations on leukocyte interferon before and after treatment with glycosidases are shown in Figs. 2 and 3. After enzyme treatment (glycosidases and a-mannosidase), the apparent molecular weight of the interferon is decreased by about 4,200. This observation was verified in three separate experiments. The molecular weight
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determined for untreated interferon is 26,000 ± 1,200. It should be noted, however, that glycoproteins are not believed to behave as globular proteins on gel filtration (14). In addition, since losses of interferon activity occurred during gel filtration in neutral buffers, our columns were eluted under acid conditions which may cause some protein unfolding. Therefore, we stress that the change in molecular weight of interferon is the important observation rather than the absolute molecular weight values.

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