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Hydrophobicity of Neuraminidase-Treated Rabbit Interferon (39711)

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Introduction. Heterogeneity of interferon molecules in biophysical, biochemical, and biological properties seems universal in all species (1). The heterogeneous property, although little understood, may be contributed to by carbohydrate moieties of the interferon molecule, the polypeptide structure, or both (2). Using bovine serum albumin-bound Sepharose 4B affinity chromatography, Huang *et al.* first demonstrated the hydrophobic nature of interferon and that there are degrees of differences in hydrophobicity among human, mouse, and rabbit interferons (3). Interferon induced from human fibroblast cells demonstrated the greatest hydrophobic property, whereas rabbit serum interferon showed the least, according to the degree of affinity for the column (3). The terminal carbohydrate moiety of rabbit interferon is believed to be sialic acid → galactose (4). The present report demonstrates that removal of the sialic acid group from rabbit interferon by neuraminidase will affect its binding property in a bovine albumin bound Sepharose 4B column.

Materials and Methods. Induction of interferon. Rabbit interferon was produced in primary rabbit kidney cells with poly(rI):poly(rC), cycloheximide, and actinomycin D as described (5) with the following modification. After the removal of antimetabolites, the cells were replenished with medium 199 (Gibco) free from serum to minimize the content of nonspecific glycoproteins presented in serum. The medium fluid was collected 3 hr later and was dialyzed at 4° against two changes of 1 liter of phosphate-buffered saline (PBS) in 24 hr. The dialyzed fluid contained about 8000 IU of interferon/0.1 ml as assayed by a semimicro assay method (6) using the NIH reference standard. Specific activity of this interferon was about 10^5 IU/mg protein.

Neuraminidase treatment of interferon. Before the enzyme treatment, interferon was dialyzed overnight in Na-acetate buffer (0.15 *M* NaCl, 20 m*M* $CaCl_2$; pH 5.5) (4) and then treated with 25 units of neuraminidase from *Vibrio cholera* (Schwartz/Mann, Lot No. AZ 2199) at 37° for 2 hr. Interferon thus treated lost about 20% of its activity. A control interferon sample was treated with 25 units of neuraminidase, which had been heat inactivated at 80° for 2 hr. The neuraminidase used contained less than 1.0 IU/ml of proteolytic enzymatic activity measured by the method of Alkjaersig *et al.* (7).

Affinity chromatography. CNBr-activated Sepharose 4B (Pharmacia, Lot No. 1761) was swollen in 0.1 m*M* HCl for at least 1 hr before use. Bovine serum albumin (Worthington) was bound to beads according to the method of Huang *et al.* (3). More than 95% of the serum albumin was bound to the beads. The albumin-bound beads were then soaked with 100 ml of 0.1 m*M* ethanolamine (pH 8.0) for 1-2 hr at room temperature. The beads were washed with 300 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl and then alternately washed with three cycles of 150 ml of 0.1 *M* Na-acetate, pH 4.5, containing 1 *M* NaCl and 150 ml of 0.05 *M* Na-phosphate buffer, pH 8.0, containing 1 *M* NaCl according to Huang *et al.* (3). The beads were washed with 150 ml of 0.02 *M* Na-phosphate buffer, pH 7.4, containing 0.15 *M* NaCl followed by 300 ml of PBS and were then packed in a 0.9 × 20-cm column at room temperature and further washed with 30 ml of PBS before use.

Neuraminidase assay. Neuraminidase was assayed by the method of Warren (8) using bovine submaxillary mucin (Worthington) as substrate and *N*-acetyl neuraminic acid (Calbiochem) as standard.

Protein assay. The protein in the fractionated interferon samples was assayed by the fluorescamine method (9). The Lowry *et al.* method (10) was used to check the interferon in medium 199 after dialysis for resid-

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ual amino acids which may interfere with the protein measurement by the fluorescamine method. There was no significant interference of protein measurement by free amino acids in dialyzed interferon preparations when the fluorescamine method was used.

Results. Heterogeneity of rabbit interferon. A representative experiment shows that the elution pattern of the control interferon sample is shown in Fig. 1A. The protein content in the interferon sample was eluted by PBS (E_0) immediately after the void volume. About 88% of interferon activity could be found in the first several fractions eluted by E_0. The protein and interferon peak of E_0 were too close together to be distinguished. There was no protein or interferon detected in a high salt eluent containing 0.02 *M* Na-phosphate and 1 *M* NaCl at pH 7.4 (E_1). The remaining 12% of the interferon activity bound to the column was eluted with 0.02 *M* Na-phosphate, pH 7.4, containing 33% by volume of a polar reducing agent, ethylene glycol (E_2). This elution pattern of interferon activity was not affected by the quantity of interferon applied to the column. These results indicate that poly (rI):poly (rC) superinduced interferon from kidney cells has two populations, one showing hydrophilic and the other showing hydrophobic properties.

The hydrophobic nature of neuraminidase-treated interferon. Figure 1B shows the

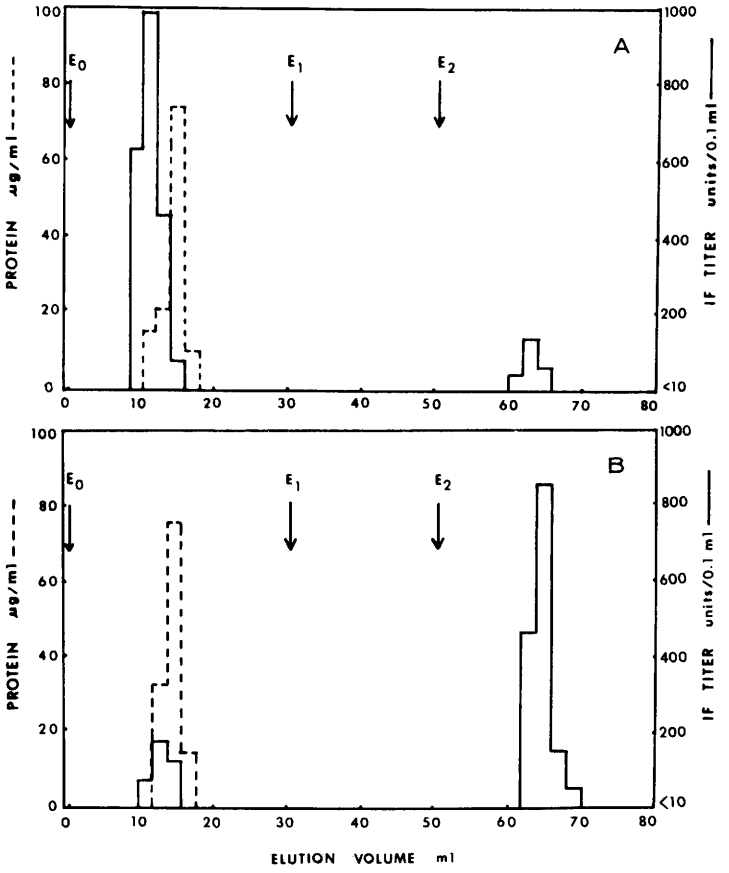

FIG. 1. Elution pattern of interferon. Two milliliters of interferon samples were layered on the column, followed by subsequent additions of 30 ml of E_0 (PBS), 20 ml of E_1 (0.02 *M* Na-phosphate, pH 7.4, containing 1 *M* NaCl), and 30 ml of E_2 [0.02 *M* Na-phosphate, pH 7.4, containing 33% (v/v) ethylene glycol]. Collection of eluents started immediately after the addition of samples. Approximately 2 ml of eluent were collected per fraction. (A) Interferon treated with heat-inactivated neuraminidase. (B) Interferon treated with neuraminidase. Protein (-----); interferon (——).

elution pattern of neuraminidase-treated interferon. After enzyme treatment, the total content of detectable protein was eluted by E_0 but only 20% of interferon activity could be found in the E_0 fractions. The majority of interferon activity (80%) had become hydrophobic and was found in fractions eluted by E_2.

Discussion. Removing sialic acid, a source of electrostatic force, from glycoproteins will increase the chances of other types of interaction of glycoprotein molecules, including a hydrophobic one. The experimental results suggest that the removal of sialic acid can affect the physical nature of interferon molecules. The finding that interferon treated with heat-inactivated enzyme showed a different affinity for albumin bound to Sepharose 4B beads indicated heterogeneity in the interferon population. After enzyme treatment, the majority of interferon molecules became hydrophobic. This change of affinity was not observed with rabbit serum interferon, probably due to the presence of abundant glycoproteins in serum which may interfere with the action of neuraminidase on interferon (data not shown). The minor peak eluted with E_0 in neuraminidase-treated interferon probably represented unaltered interferon. However, increasing the amount of enzyme to 50 units or the incubation time to 4 hr, or both, did not result in change in the elution pattern of interferon activity. The degree of neuraminidase action on interferon molecules is not known. The method we used to assay sialic acid was not sensitive enough to detect a measurable amount released from the interferon preparation. A control experiment using mucin as the substrate resulted in saturation of enzyme action under the conditions used for interferon treatment. Neuraminidase was eluted only by E_0 in a control experiment. The results suggest that the change of affinity of interferon to the albumin-bound Sepharose 4B beads after neuraminidase treatment and the difference in hydrophobicity among interferons are probably related to the content of sialic acid on interferon. Recently, Jankowski *et al.* (2) suggested that human leukocytic interferon is not a glycoprotein and is not hydrophobic, whereas the glycosylated human fibroblast interferon is hydrophobic. If the content of sialic acid is proportional to the content of carbohydrates in interferon, these results seem to be contradictory to the present observation. The contradiction cannot be explained at this time. However, very recently, carbohydrates were found in human leukocytic interferon (11). It remains to be shown whether the removal of the charged group, sialic acid per se is responsible for the change in affinity or whether removal of sialic acid causes consequent changes in the polypeptide conformation which then are responsible for the change of affinity.

Summary. Poly(rI):poly(rC)-superinduced rabbit interferon can be separated by elution into two populations in a bovine serum albumin-bound Sepharose 4B column by phosphate-buffered saline (E_0) and by an eluent containing a polar reducing agent (E_2). Approximately 88% of interferon activity is eluted with E_0, along with all detectable protein, while there is about 12% of interferon activity bound to the column which can only be eluted with E_2. After interferon is treated with neuraminidase, it is found that the majority of the activity (80%) is bound to the column and can only be eluted with E_2. Only 20% of the activity is found in the E_0 fractions, which also contain all measurable protein. The sialic acid residue of interferon may therefore be related to the hyperphobicity of the molecules.

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