Use of Glycosyltransferases to Restore Pertussis Toxin Receptor Activity to Asialoagalactofetuin*

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Pertussis toxin activates a non-specific adenylate cyclase, and the 125I-labeled to pertussis toxin, coated polystyrene tubes. Fetuin oligosaccharides were sequentially degraded by treatment with: neuraminidase (asialofetuin) followed by β-galactosidase (asialoagalactofetuin) and, lastly, with β-N-acetylgalactosaminidase (asialoagalacto-a[N-acetylgalactosaminofetuin]). Asialofetuin retained only 19 and 53% of the inhibitory activity of native fetuin in the hemagglutination and 125I-fetuin binding assays, respectively. Asialoagalactofetuin showed no further reduction in inhibition in the hemagglutination system and, instead, resulted in partial recovery of inhibition in the 125I-fetuin-pertussis toxin binding assay. Asialoagalacto-a[N-acetylgalactosaminofetuin showed a further decrease in ability to inhibit pertussis toxin binding in both assays. The inhibitory activity of asialoagalactofetuin could be restored to that of native fetuin by adding back β-galactosyl-1,4-β-galactosyltransferase, followed by the addition of terminal sialic acid residues with CMP-N-acetylneuraminic acid:β-d-galactosyl-1,4-N-acetyl-β-d-glucosamine-α-2,6-N-acetylneuraminyltransferase. The data suggested that a requirement for pertussis toxin binding to fetuin may be the presence of acemann-containing sugar groups in the nonreducing terminal position of fetuin’s oligosaccharides.

Pertussis toxin is one of the virulence factors produced by Bordetella pertussis, the etiological agent of whooping cough (Muñoz, 1985). It is the component best correlated with protection in the current whole cell pertussis vaccine (Muñoz et al., 1981; Sato and Sato, 1984), and most likely responsible for the neurological sequelae sometimes associated with the disease and in a small number of those vaccinated (Miller et al., 1981; Steinman et al., 1985). Consequently, there is considerable interest in elucidating structure-function relationships in pertussis toxin with a view to understanding its role in the disease process and for producing a safer acellular vaccine preparation.

Pertussis toxin provides another example of an A-B class of toxin (Gill, 1978). It is a heterohexameric protein consisting of five subunits, designated S1 to S5 (Peppler et al., 1985).

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Effect this has on binding activity. However, the information gained from this approach is limited because: (a) receptor recognition and activity are stereospecific, the sugar's glycosidic linkage (Kronis and Carver, 1985; Wright, 1984); and (b) the specificity of exoglycosidase enzymes is not restricted to particular oligosaccharide sequences, a number of which are present in fetuin (Edge and Spiro, 1987; Spiro and Bhoyroo, 1974; Takasaki and Kobata, 1986; Townsend et al., 1986). However, the missing information can be obtained by using glycosyltransferases to add sugars back, in defined linkages, to known acceptor groups on exoglycosidase-trimmed oligosaccharides, thereby restoring receptor activity (Carroll et al., 1981; Rogers and Paulson, 1983). This approach not only allows the investigation of receptor structure and function at the primary chemical level, it has the advantage of maintaining the natural molecular environment for the restructured oligosaccharide units.

The glycosyltransferases used in this study were: galactosyltransferase (UDP-D-galactose: D-glucose-1-galactosyltransferase) which, in the absence of a-lactalbumin, catalyzes the general glycosyltransferase reaction (Khatra et al., 1974):

\[
\text{UDP-Gal} + \text{GlcNAC} \rightarrow \text{UDP} + \text{Gal(b1-4)GlcNAC}
\]

and Gal(b1-4)GlcNac(2-6) sialyltransferase which exhibits strict specificity for acceptor substrates containing the Gal(b1-4)GlcNac sequence and transfers NeuAc from cytidine-5'-monophospho-N-acetylneuraminic acid to the trisaccharide NeuAc(a2-6)Gal(b1-4)GlcNac (Van Den Eijnden et al., 1980; Weinstein et al., 1982b).

Experimental procedures

Materials—The following reagents were obtained from Sigma: fetuin (type IV) from fetal calf serum, fetuin-agarose, fraction V bovine serum albumin (BSA), 1 human a-1-acid glycoprotein (purified from Cohn fraction VI), human haptoglobin, neuraminidase (EC 3.2.1.18) from Clostridium perfringens, b-D-galactosidase (EC 3.2.1.23) from bovine testes, b-N-acetyl-b-hexosaminidase (EC 3.2.1.52) from jack beans, UDP-galactose: b-1,4-b-D-galactosyltransferase (EC 2.4.1.22) from bovine milk, the sodium salt of uridine-5'-diphospho-galactose (UDP-galactose), and the ammonium salt of cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-neuraminic acid), 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (neuraminidase inhibitor). The specificity of the exoglycosidases were confirmed using the p-nitrophenylglycoside derivatives of the appropriate carbohydrate substrates (obtained from Sigma) for each enzyme. Colomnic acid, human milk neuraminidase, tri-N-acetylchitotriose, bi-N-acetylchitobiose, synthetic N-acetylstarchose, and N-glycolyl-neuraminic acid were also obtained from Sigma. Other sugars were obtained from Supelco Canada Ltd. (Oakville, Ontario). CMP-N-acetylneuraminic acid-Galactosyl-1,4-N-acetyl-b-D-glucosamine-2,6-N-acetylneuraminyltransferase (2,6-sialyltransferase; EC 2.4.99.1) isolated from rat liver was obtained from Boehringer Mannheim Co., Dorval, Quebec. Pertussis toxin was obtained from List Biological Laboratories Inc., Campbell, CA. Goose blood was from Gibmar Laboratories, Argrossan, Alberta, Canada. Na2SO4 was obtained from Edmonton Radiopharmaceuticals Inc., Edmonton, Canada. Methanolic HCl and trifluoroacetic anhydride were obtained from the Pierce Chemical Co.

Buffers—Buffer A: 0.1 M sodium phosphate-buffered physiological saline (0.15 M NaCl; pH 7.2) containing 1 mM CaCl2 and 1 mM MgCl2. Buffer B: 0.1 M citric acid, 0.2 M sodium phosphate buffer (pH 4.5). Buffer C: 50 mM MOPS (pH 7.4) containing 10 mM MnCl2, 0.15 M NaCl, Buffer D: 25 mM sodium cacodylate (pH 6.0) containing 50 mM NaCl. Buffer E: 15 mM NaCO3, 35 mM NaHCO3 (pH 8.3). Buffer F: 5 mM sodium phosphate (pH 7.5) containing 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (added immediately prior to use). All buffers contained 0.1% sodium azide as an antimicrobial agent for procedures requiring extended periods of incubation at 37 °C.

Treatment of Fetuin with Exoglycosidases—One hundred mg of fetuin was dissolved in 4 ml of buffer B, and the solution was transferred to a 12 × 75-mm capped polystyrene culture tube. A portion (1 ml) of this solution was set aside in a separate culture tube for the control. Forty milliliters of neuraminidase (250 µg) was then added to all remaining solutions. The tubes were incubated for 18 h at 37 °C while slowly turning (5 rpm) in an end-over-end rotator. After an additional 40 milliliters of fresh neuraminidase was added, the samples were incubated for an additional 18 h at 37 °C to complete the desialation reaction. Next, the samples were diluted 20 times with buffer B, and 0.4 units (250 µg) of neuraminidase was added. The samples were then concentrated 20-fold using an Amicon Model 8010 ultrafiltration apparatus fitted with a 25-mm diameter YM-10 filter (molecular weight 10,000 cutoff) and incubated for 72 h at 37 °C to remove galactose residues. The samples were then diluted again 20 times with buffer B, and 1 unit (130 µg) of jack bean b-N-acetylgalactosaminidase was added and the mixture concentrated and incubated at 37 °C for 72 h to remove N-acetyhexosaminic groups. Next, the samples were diluted at least 20-fold in buffer A and concentrated in the Amicon ultrasift. This procedure was repeated three times to remove any remaining sugars which had been released from protein by the exoglycosidase treatments. In each step a random sample was removed and set aside for sugar analyses, protein concentration determination, and the binding-inhibition assays. The derivates were stored at -20 °C until use.

Treatment of Asialo-galactofetuin with Glycosyltransferase—A portion (1 mg) of the asialo-galactofetuin was diluted to 20 ml in buffer C and concentrated to 0.5 ml in the Amicon ultrafiltration unit as described above. This procedure was repeated. Next, 1.25 units of galactosyltransferase (313 µg) and 2 mg UDP-galactose (2 mol of UDP-Gal/mol of GlcNAc acceptor groups) were added, and the mixture was incubated for 24 h at 37 °C. A portion of the sample was removed for the hemagglutination inhibition and sugar analyses, and the remainder was diluted 20 times in buffer D. This sample was concentrated, and 10 milliliters of the sialyltransferase (1.25 µg) and 2 mg of CMP-N-acetylneuraminic acid (approximately 2 mol of CMP-NeuAc/mol of Gal acceptor groups) were added. After incubation for 24 h at 37 °C the reaction mixture was diluted 20 times in buffer A and concentrated to 0.5 ml. This final step was performed three times to ensure that all of the free monosaccharides were removed from the fetuin solution. Each of the aglycofetuin derivatives were diluted 25 times in buffer A to a protein concentration of roughly 0.5 mg/ml. The protein concentrations of the inhibitors was determined by the Lowry procedure (Lowry et al., 1951) using native fetuin for the standard. The protein concentrations were confirmed spectrophotometrically using an extinction coefficient of 4.1 for a 1% solution at λ 278 nm (Spiro, 1960).

Sugar Analysis by Gas-Liquid Chromatography—Sugar analyses were done as liquid-gas chromatography (GLC) of the trifluoroacetate derivatives of the O-methyl glycosides essentially as described previously (Zanetta et al., 1972). The analyses were performed on a Varian Vista 6000 (Varian Associates, Sunnyvale, CA) gas chromatograph equipped with a flame-ionization detector and a fused silica capillary column (30 m × 0.25 mm inner diameter) with a bonded and cross-linked nonpolar liquid phase 0.25 µm thick (a DB-5 column from J & W Scientific Co., Rancho Cordova, CA). Injection was performed in the splitless mode (0.5 min) followed by a 100:1 split flow. The carrier gas (helium) had a linear velocity of 42 cm/s at the starting temperature of 90 °C. The column temperature was maintained at 90 °C for 1 min after the injection and then increased to increase at a rate of 8 °C/min to 270 °C. The injector and detector temperatures were 260 and 272 °C, respectively. Data processing was performed with a Varian 401 chromatograph data system. The relative amount of each sugar in the exoglycosidase-treated fetuin derivatives was obtained by comparing the area of each sugar peak relative to the area of the mannose peak in each sample. The reduction in sugar content in the aglycofetuin derivatives was expressed as the percentage of the mannose sugar ratios obtained for each derivative as compared with those obtained for native fetuin. Sugars were identified by comparison with authentic samples obtained from Suclope. To prepare the samples for analysis by GLC, fetuin and the aglycofetuin samples were diluted in 20 volumes of distilled water and concentrated in an Amicon Model 3 micro-ultrafiltration apparatus as described earlier. This procedure was repeated three times to remove most of the buffer salts and low molecular weight carbohydrate material (free sugars). Samples (200–500 µg) were then

1 The abbreviations used are: BSA, bovine serum albumin; MOPS, 3-(N-morpholino)propanesulfonic acid; GLC, gas-liquid chromatography.
transferred to 1.5-ml amber, screw-cap septum vials (Fisher Chemical Co.) which had been rinsed with acetonitrile and dried with N₂ gas prior to use. Next, the samples were dried in a Savant Spin-Vac concentrator (Model SVC-100H, Savant Instruments Inc., Hicksville, NY) and dissolved in 50 µl of 2.0 N methanolic HCl (3 N methanolic HCl from Supelco, diluted 2:1 in anhydrous methanol prior to use). The vials were purged with Nz gas, tightly closed with Teflon-lined screw caps, and placed in a heating block at 85 °C. After the initial warm-up period (~5 min), the vials were shaken vigorously with a Vortex mixer to ensure complete dissolution of the sample, and the caps were tightened further. Methanolysis was allowed to continue for 18 h at 85 °C. The caps were then removed from the cooled sample vials and the methanolic HCl was evaporated at 45 °C in a gentle stream of Nz gas. One hundred µl of trifluoroacetic anhydride:acetonitrile (1:1) was added, and the vials were sealed and incubated at 85 °C for 1 h. The samples were cooled, thoroughly dried in a stream of dry nitrogen, dissolved in 100 µl of anhydrous acetonitrile, and 1–2 µl was injected into the chromatograph.

Goose Hemagglutination Inhibition Assay—Pertussis toxin was suspended to a concentration of 1 µg/ml (by weight protein) in buffer A and then mixed with a Branson Model B-220 ultrasonic water bath, We found it necessary to sonicate diluted pertussis toxin solutions before each experiment to obtain consistent hemagglutination titers. Next, serial 2-fold dilutions of the inhibitor solutions were prepared in buffer A, and 50 µl of each dilution was added to the round bottom wells in 96-well microtiter plates. Fifty µl of sonicated pertussis toxin solution was then mixed with the inhibitors and the plates were incubated for 30–45 min at room temperature to allow binding to occur. The plates were then washed and incubated at 65 °C for 1 h. The samples were cooled, thoroughly dried in a stream of dry nitrogen, dissolved in 100 µl of anhydrous acetonitrile, and 1–2 µl was injected into the chromatograph.

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**RESULTS AND DISCUSSION**

Diagrams of fetuin's N- and O-linked oligosaccharides are presented in Fig. 1 to illustrate the diversity of structural features which are available for interacting with the pertussis toxin binding site. In addition to the major differences between the two classes of oligosaccharide, there are minor differences which contribute to structural heterogeneity within each class (Edge and Spiro, 1987; Spiro and Bhoyroo, 1974; Takasaki and Kobata, 1986; Townsend et al., 1986). For example, approximately 17% of fetuin's N-linked oligosaccharides differ in the GlcNAc(β1,4)-Man(β1,3)-linked antennae which contains a Gal glycosidically attached to GlcNAc in the (β1,3) instead of (β1,4) linkage. Three O-linked structures have been identified (Edge and Spiro, 1983; Spiro and Bhoyroo, 1974); a hexa-, tetra-, and trisaccharide. The O-linked hexaasaccharide represents a branched structure in which one of the NeuAc(α2,3)Gal arms is attached to GlcNAc which in turn is (β1,6)-linked to the reducing terminal GalNAc group. The other NeuAc(α2,3)Gal arm is (β1,3)-linked directly to the terminal GalNAc. Moreover, the GlcNAc (β1,4)-linked to Man and the O-linked GalNAc sugars may also contain internal (α2,6)-linked NeuAc, the majority of which are (α2,3)-linked to terminal Gal in both oligosaccharide classes.

To investigate which portions of the structures shown in Fig. 1 were important for pertussis toxin binding, exoglycosidase and glycosyltransferase enzymes were used to prepare aglycofetuin derivatives for use in the binding activity assays. The sugar content of the aglycofetuin derivatives was determined by GLC, the results of which are shown in Table I and Fig. 2 accompanied by drawings of the predicted structures of the modified oligosaccharide units. Although neuraminidase and bovine testes β-galactosidase removed 92 and 87% of fetuin's NeuAc and Gal groups, respectively, treatment of asialoagalactofetuin with jack bean β-N-acetylhexosaminidase caused a decrease of 70% in the hexosamine peak area relative to the peak area of mannose (Table 1). However, under the methanolysis conditions used, the N-acetylgalactosaminylasparagine bond was probably only partially cleaved (Chambers and Clamp, 1971); and, accordingly, all of the asparagine-linked GlcNAc groups would not have registered on the chromatograms. In addition, only four of the remaining five terminal N-acetyhexosaminylose residues are in the β-linkage suitable for enzymatic attack. Thus, a less than complete decrease in the area of the N-acetyhexosamine peak was expected and, the data obtained from analysis of the GLC results is consistent with the suggestion that jack bean N-acetylhexosaminidase had released all of the terminal GlcNAc groups from asialoagalactofetuin's N-linked oligosaccharides and the small amount of GlcNAc present in the O-linked hexaasaccharide.

When asialoagalactofetuin was treated with galatosyltransferase, the amount of Gal increased from 6 to 64% of native levels (Table I and Fig. 2). In native fetuin, approximately 75% of the Gal residues are bound in the (β1,4) linkage to GlcNAc on the N-linked oligosaccharides, and approximately 25% are attached to O-linked GalNAc groups. In addition, a small number of Gal groups are attached to O-linked GlcNAc (Fig. 1). Since galatosyltransferase only adds Gal in the (β1,4) linkage to GlcNAc acceptor groups (Khatra et al., 1974), the enzyme had apparently attached Gal onto approximately 85% of the available acceptor groups in the asialoagalactofetuin. Therefore, all but 15% of the antennae in the oligosaccharides of the galatosyltransferase-treated asialoagalactofetuin now terminated in Gal(β1,4)GlcNAc, the proper acceptor for the 2,6-sialyltransferase. The remaining N-linked antennae terminated in GlcNAc, the (β1,3)-linked Gal groups found in native fetuin were no longer present, and, except for a small amount of Gal(β1,4)GlcNAc linked to GalNAc (not shown in Figs. 2 and 3), the GlcNAc residues were all that remained of the majority of O-linked sugars.

Exposure of the galatosyltransferase-treated asialoagalactofetuin to 2,6-sialyltransferase in the presence of CMP-NeuAc restored almost 40% of the native level of NeuAc groups (Table I and Fig. 2). Considering the standard error...
A. N-LINKED OLIGOSACCHARIDES

\[ \text{NeuAc} \] (α 2-3) Gal (β 1-4) GlcNAc (β 1-2) Man (β 1-6) 
\[ \text{Man} \] (β 1-4) GlcNAc (β 1-4) GlcNAc (β 1-N)ASN 
83% of all N-linked

\[ \text{NeuAc} \] (α 2-3) Gal (β 1-4) GlcNAc (β 1-2) Man (β 1-3) 
\[ \text{NeuAc} \] (α 2-6) Gal (β 1-3) GlcNAc (β 1-4) Man (β 1-3) 
\[ \text{NeuAc} \] (α 2-6) Gal (β 1-4) GlcNAc (β 1-2) 
17% of all N-linked

B. O-LINKED OLIGOSACCHARIDES

\[ \text{NeuAc} \] (α 2-3) Gal (β 1-3) GalNAc (β 1-O)SER (α THR) 70% of all O-linked

\[ \text{NeuAc} \] (α 2-6) Gal (β 1-3) GalNAc (β 1-O)SER (α THR) 23% of all O-linked

\[ \text{NeuAc} \] (α 2-3) Gal (β 1-4) GlcNAc (β 1-5) 
\[ \text{NeuAc} \] (α 2-3) Gal (β 1-O)SER (α THR) 7% of all O-linked

Fig. 1. Structures of fetuin N- and O-linked oligosaccharides as determined by Takasaki and Kobata (1986), Spiro and Bhoyroo (1974), and Edge and Spiro (1987). The relative proportions of each of the structures is also indicated. For clarity, the monosaccharides in each of the oligosaccharide units have been assigned symbols in order to draw the structural diagrams which identify the aglycofetuin derivatives in Figs. 2 and 3. The sialic acids in fetuin were previously identified as NeuAc (Takasaki and Kobata, 1986). The different glycosidic linkages of the important NeuAc and Gal groups are indicated by the orientation of the symbols for these sugars.

and, for the present, ignoring the small amount of O-linked Gal(β 1-4)GlcNAc acceptor groups, the data displayed in Table I suggested that the sialyltransferase had added NeuAc to only one of the three potential acceptor sites generated by the galactosyltransferase on the N-linked oligosaccharides. Although a previous study by Weinstein et al. (1982b) indicated that the rat liver 2,6-sialyltransferase used in our study should have been capable of attaching NeuAc to all of the available (β 1,4-linked Gal acceptor groups on the tetraantennary oligosaccharide units of α1-acid glycoprotein, van den Eijnden et al. (1980) demonstrated that the 2,6-sialyltransferase from bovine colostrum apparently prefers attaching NeuAc to Man(α 1-3) antennae in triantennary oligosaccharides such as those found on fetuin. These contrasting results may be due to differences in the acceptor specificities of enzymes from two different sources. However, another explanation could be that the tetraantennary oligosaccharide units of α1-acid glycoprotein may assume a different spatial configuration than fetuin's triantennary oligosaccharides. Such a difference could also contribute to the preferential action of the rat liver sialyltransferase on one of fetuin's three, N-linked antennae. Alternatively, our inability to achieve restoration of NeuAc to all available acceptor sites may have been due to the presence of residual neuraminidase activity in the aglycofetuin preparations.

To resolve this issue we have prepared asialofetuin and asialo-α1-acid glycoprotein using a procedure designed to reduce the possibility of neuraminidase contamination (Weinstein et al., 1982a). As an extra precaution, we have performed the resialation reactions in the presence of the neuraminidase inhibitor 2,3-dehydro-2-deoxy-N-acetylenuraminic acid (Reutter et al., 1982). Under these conditions, the rat liver sialyltransferase was able to restore 91 ± 12% (n = 4) of the sialic acid to asialo-α1-acid glycoprotein but only 55 ± 6% (n
Our results also suggested that pertussis toxin possessed lectin-like properties similar to those of wheat germ agglutinin which binds to terminal GlcNAc and NeuAc residues in complex oligosaccharides (Kronis and Carver, 1985; Wright, 1984). However, wheat germ agglutinin apparently prefers binding to \((\alpha 2,3)\)-linked NeuAc groups in oligosaccharides (Kronis and Carver, 1985), whereas our data suggested that pertussis toxin may prefer binding to terminal \((\alpha 2,6)\)-linked NeuAc groups. We were not able to test the activity of fetuin derivatives containing the other NeuAc linkage configurations because the necessary sialyltransferase enzymes were not available for the study. However, a panel of sugars and glycosides was screened to further investigate the linkage and sugar specificity of pertussis toxin receptors. The following sugars did not inhibit the goose hemagglutination reaction (unless otherwise noted the final concentration of the sugars was 100 mM): L-glucose, \(\alpha\)-D-glucose, \(\beta\)-D-glucose, glucosamine, \(N\)-acetylglucosamine, \(N\)-acetylgalactosamine, methyl-\(\alpha\)-D-glucopyranoside, 2-deoxy-D-glucose, glucose, tyrosine, phenyl-\(\beta\)-D-glucopyranoside (50 mM), arabinose, salicin, galactose, lactosamine, lactitol, mannose, N-mannosamine, methyl-\(\alpha\)-D-mannopyranoside, L-fucose, fructose, L-rhamnose, D-arabinose, D-arabitol, L-xylitol, D-xylitol, methyl-\(\alpha\)-D-xylpyranoside, methyl-\(\beta\)-D-xylopyranoside, D-ribitol, 2-deoxy-D-ribose, L-sorbine, sorbitol, lyxose, meso-erythritol, lactose, \(N\)-acetyllactosamine, maltose, sucrose, trehalose, melibiose, cellobiose, turanose, raffinose, \(N\)-glycolylneuraminic acid, \(N\)-aminic acid, colominic acid, and tri-\(N\)-acytethylchitotriose (25 mM). In contrast, chitobiose and neuraminic acid from human milk inhibited pertussis toxin-mediated hemagglutination at a minimum concentration of 50 mM each. The observation that chitobiose inhibited hemagglutination is consistent with the wheat germ asialo-like activity of pertussis toxin. However, the inhibitory effect was only noticed at concentrations greater than 50 mM. Chitotriose did not inhibit hemagglutination, but it was not possible to test concentrations greater than 25 mM due to the limited solubility of this trisaccharide. Furthermore, the observation that human milk-derived neuraminic acid, but not NeuAc alone, lactose, or \(N\)-acetyllactosamine inhibited hemagglutination also supported our suggestion of the importance of \(N\)-acetyllactosamine-linked NeuAc groups for pertussis toxin binding to fetuin. However, because the neuraminic acid contained both \((\alpha 2,3)\)- and \((\alpha 2,6)\)-linked NeuAc we were unable to use this material to investigate preferential binding of pertussis toxin to one versus the other NeuAc isomer. Nonetheless, it is important to note that none of the inhibitory sugars prevented the hemagglutination reaction at concentrations approaching the minimum inhibitory concentration of fetuin (0.3 ± 0.2 mM; \(n = 11\)).

Although the contribution of NeuAc groups to binding appeared to be greater than that of GlcNAc in the hemagglutination inhibition assay (Fig. 3), the \(1^2\) -fetuin binding inhibition studies suggested that the two sugars were of equal structural importance. In the \(1^2\) -fetuin binding inhibition system, neuraminidase treatment reduced native fetuin's activity by approximately 47%, and, in comparison to the activity of asialoagalactofetuin, \(N\)-acetyllactosaminidase caused a 54% decline in activity (Fig. 3). The different behavior of the fetuin derivatives in the two assay systems may indicate differences between the way that pertussis toxin interacts with the oligosaccharide domains of fetuin and receptors present in the membranes of goose erythrocytes.

Pertussis toxin receptor activity of glycoproteins may be modulated by factors other than the structure of the oligosaccharides.

### Table I: Results of GLC analysis of aglycofetuin derivatives

| Fetuin derivative | Sugar | \(\text{peak area ratio} \pm \text{S.E.}\) | % Native ratio \(\pm \text{S.E.}\) |
|-------------------|-------|-------------------------------------------|----------------------------------|
| Native fetuin     | NeuAc | 6 \(1.7100 \pm 0.1673\) 100 |                                  |
|                   | Gal    | 6 \(0.7213 \pm 0.0322\) 100 |                                  |
|                   | HexNAc | 6 \(1.2367 \pm 0.0351\) 100 |                                  |
| Asialoagalactofetuin | NeuAc | 4 \(0.1389 \pm 0.0183\) 8.10 ± 0.39 |                                  |
|                   | Gal    | 4 \(0.0932 \pm 0.0267\) 12.78 ± 4.43 |                                  |
|                   | HexNAc | 4 \(1.1481 \pm 0.0570\) 103.39 ± 17.81 |                                  |
| Asialoagalacto-  | NeuAc | 3 \(0.1270 \pm 0.0242\) 7.36 ± 0.98 |                                  |
| AxNAC-fetuin      | Gal    | 3 \(0.0404 \pm 0.007\) 5.57 ± 1.02 |                                  |
|                   | HexNAc | 3 \(0.3701 \pm 0.0481\) 30.08 ± 4.29 |                                  |
| + Galactotransferase | NeuAc | 2 \(0.0847 \pm 0.0641\) 4.63 ± 4.66 |                                  |
|                   | Gal    | 2 \(0.4698 \pm 0.0534\) 63.84 ± 14.28 |                                  |
|                   | HexNAc | 2 \(0.9272 \pm 0.1907\) 74.61 ± 18.24 |                                  |
| + Galactosyl- + sia- | NeuAc | 3 \(0.6749 \pm 0.0128\) 39.78 ± 4.44 |                                  |
| lytransferase     | Gal    | 3 \(4.1586 \pm 0.0015\) 57.34 ± 3.33 |                                  |
|                   | HexNAc | 3 \(1.0496 \pm 0.0531\) 84.77 ± 2.67 |                                  |
Fig. 2. Analysis of carbohydrates in exoglycosidase-treated fetuin derivatives by gas-liquid chromatography. Gas liquid chromatography was performed on the trifluoroacetate derivatives of O-methyl glycosides as described in the text. Only the major isomers of the different monosaccharides are labeled. Peaks (●) were identified as residual buffer components, and the structural diagrams are composites of those shown in Fig. 1. Brackets surrounding the (α2-6)-linked NeuAc symbol in the galactosyltransferase and sialyltransferase-treated derivative indicate that its assignment to the (α1-3)Man-linked antenna is tentatively based on its position in native fetuin and the studies of van den Eijnden et al. (1980).

charide sequences. This could provide an explanation for the observation that it required much higher concentrations of neuraminlactose than fetuin to inhibit pertussis toxin-mediated hemagglutination. The number (valence) of glycosylated sites on glycoprotein receptors, the number of antennae in branched oligosaccharide units, or structural components of the core sugars or peptide sequences may also contribute to binding activity. For example, although the tetraantennary oligosaccharides of α1-acid glycoprotein contain the NeuAc (α2-6)Gal(β1-4)GlcNAc sequence (Montreuil, 1984), we found that the minimum pertussis toxin hemagglutination inhibitory concentration of α1-acid glycoprotein was 13.5 ± 0.5 μM (n = 4). Conversely, although each fetuin molecule contains only three asparagine-linked oligosaccharides and α1-acid glycoprotein contains five to eight (Jeanloz, 1972; Takasaki and Kobata, 1986; Townsend et al., 1986), the average minimum pertussis toxin hemagglutination inhibitory concentration for fetuin was approximately 45 times better (0.3 ± 0.2 μM; n = 11) than that of α1-acid glycoprotein. Moreover, we have also observed that proteolytic degradation of fetuin also greatly reduced its ability to bind to pertussis toxin (minimum pertussis toxin hemagglutination inhibitory concentration > 10 μM; n = 5). Therefore, we are presently characterizing the pertussis toxin receptors in goose erythrocyte membranes so that it will be possible to determine their relationship to fetuin. We feel that the difference observed in the abilities of α1-acid glycoprotein, fetuin glycopeptides, and human neuraminlactose to inhibit hemagglutination provides evidence that the interaction of pertussis toxin with native fetuin (and possibly with receptors on cell surfaces) is dependent on the correct spatial orientation of the important sugar groups rather than on electrostatic interactions with...
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Fig. 3. Activity of fetuin derivatives in the goose hemagglutination inhibition (stippled bars) and polystyrene tube binding inhibition (striped bars) assays. The assays were performed as described in the text. The structural diagrams are the same as those shown in Fig. 2. The error bars represent the standard deviation of the mean value of the data obtained from at least three independent trials.

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