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Analysis of pigeon intestinal mucin allergens using a novel dot blot assay

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Abstract

Many glycoproteins contain multiple glycosylation sites that can present multi-valent epitopes for antigenic recognition. Release of the oligosaccharides results in loss of avidity of antibody binding, which has been overcome by reforming clustered ligands, usually by reductive amination of free reducing oligosaccharides to poly-amine groups. We have adapted this approach to hydrazinolytic release of O-linked chains of mucin glycoproteins and 'one-pot' microscale coupling to poly-L-lysine (PLL). The conjugated PLL adheres to nitrocellulose membranes through washing procedures required for antibody or lectin overlay and detection. We show evidence for the applicability of this technique using lectin and antibody reactivity to the oligosaccharides of pigeon intestinal mucins, which have been implicated in the allergic disease pigeon fanciers' lung.

Keywords: Dot blots; Antibodies; Lectins; Hydrazinolysis; Mucin; Allergy

1. Introduction

In order to carry out structural and antigenic analysis of the oligosaccharide chains of multiply glycosylated proteins, it is necessary to release the oligosaccharides and then couple each purified form to a reagent for high-sensitivity analysis. Coupling of oligosaccharide via reductive amination to proteins [1,2], to poly-L-lysine (PLL), poly-acrylamide or stearylamine [3,4], to phosphatidyl ethanolamine [5,6] and to fluorescent labels [7–9] is a common reaction in glycobiology. The technique was first advanced for vaccine production against oligosaccharide antigens [10] and then for characterisation of anti-carbohydrate antibody specificity, before adoption for sensitive chromatographic analysis [7–9]. For characterisation of the antigenicity of glycoproteins or polysaccharides, release of oligosaccharides results in the loss of their multivalency required for high-avidity interactions and therefore the above strategies have involved attachment of purified oligosaccharides to clustered amine groups.

In order to screen for antigenicity and lectin activity to multiple oligosaccharides released from pigeon intestinal mucin by hydrazinolysis, we have set up a micro-scale coupling of

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oligosaccharides to PLL, which provides a multivalent molecule that adheres to dot blots throughout washing procedures required for antibody or lectin overlay. This is a convenient 'one-pot' reaction. Pigeon intestinal mucin is a major antigen in the allergic disease pigeon fanciers' lung (PFL) [11,12]. It has been suggested that antibody responses to epitopes on the oligosaccharides of the highly glycosylated regions of this antigen are important in the development of disease [11,13]. The present paper reports verification of the procedure by lectin and antibody overlay as a structural and antigenic screen before purification and detailed studies of active oligosaccharides.

2. Results

Preliminary oligosaccharide analysis of pigeon gastrointestinal mucin had shown a monosaccharide composition characteristic of mammalian mucin glycoproteins. In this study therefore, the oligosaccharide chains were released by hydrazinolysis under the conditions for O-glycans at 60 °C for 4 h [14]. After purification, as discussed in Section 4, the oligosaccharides were fractionated by HPLC on a reversed-phase, porous graphitised carbon (PGC) column [15,16]. One aliquot of the fractions was taken for analysis of total hexose (Fig. 1) and a second aliquot was reacted with PLL and then spotted onto nitrocellulose membranes. The conditions for optimum coupling of oligosaccharides to PLL were worked out by experiments with lactose and sialyl lactose molar concentrations 10:1 with respect to PLL; at pH 7.4, 8.7 and 9.5; for 4, 8, 18, 40 and 48 h; and at 25, 35 and 55 °C. The maximum coupling (75%) was at pH 7.4 for 4–18 h at 35 or 55 °C. After the reaction as described in Section 4, the reaction mixture was neutralised with acetic acid and evaporated and re-evaporated with methanol before dialysis and analysis of retained, coupled oligosaccharide by hexose assay.

For the pigeon mucin oligosaccharide–PLL conjugates, the nitrocellulose membranes were overlaid with lectins or sera from pigeon fanciers as discussed in Section 4. The results for two antisera are shown in Fig. 2(A and B) and for two of the lectins, AAL and MAL-1, respectively, in Fig. 2(C and D). Although the major hexose-containing fraction is 26 (Fig. 1), the blot corresponding to this (Fig. 2) is not necessarily that with the greatest staining for the lectins or antibodies. Thus the antibody binding in Fig. 2(A) and the lectin binding in Fig. 2(C) show more binding with Fraction 28. Further, they show a similar

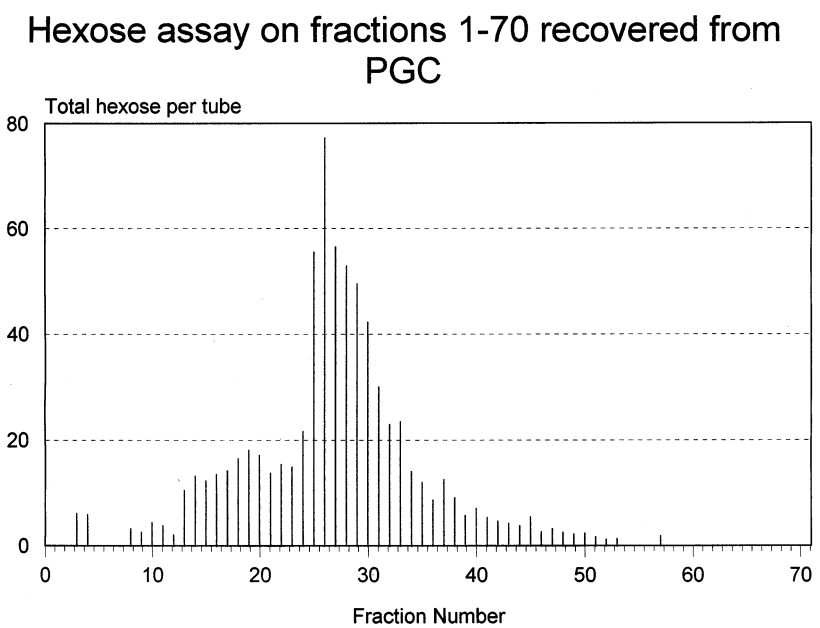

Fig. 1. Hexose assay of the fractions separated by PGC-HPLC of oligosaccharides released from pigeon intestinal mucin. Fractions were collected every minute at a flow rate of 1 mL/min.

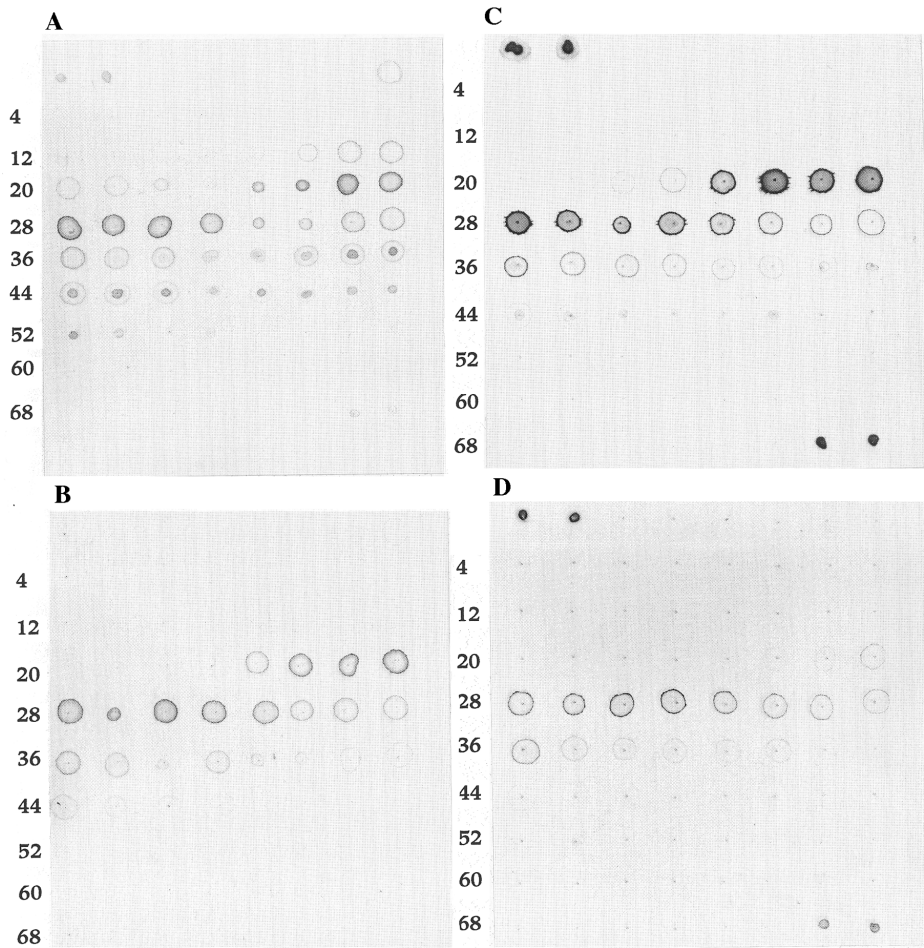

Fig. 2. Dot blots with two anti-sera from patients with PFL (A and B) and two lectins AAL (C) and MAL-1 (D). Fractions from PGC-HPLC 4-68 are indicated. In addition, horizontally along the top row are the original pigeon intestinal mucin 1 μL of 100 μg/mL in PBS (× 2); PBS (× 1), PLL (× 2); the void volume (× 2) and the solvent front (× 1) of the PGC-HPLC. Horizontally along the bottom row are fractions 68–70; PBS (× 1); PLL (× 2); and, original pigeon intestinal mucin 1 μL of 100 μg/mL in PBS (× 2).

binding pattern, whereas the antibody in Fig. 2(B) and the lectin in Fig. 2(D) show similar binding to each other, but somewhat different from those in Fig. 2(A) and Fig. 2(C). This is important because it shows in one relatively simple assay that the antibodies present in different patients with PFL have different reactivities and that these are most likely to be against O-linked sugar specificities, the first (Fig. 2(A)) for oligosaccharides having terminal Fuc(α1-3/4) and the second (Fig. 2(B)) for oligosaccharides having terminal Neu5Ac. The antibodies show additional reactivities for the larger oligosaccharides that are present in lower amounts by hexose assay. Another 13 sera were tested, giving similar results to the two patterns shown in Fig. 2. Sera from two normal controls did not react with any of the fractions, or the mucin positive control, at dilutions of 1/500 or 1/5000 (data not shown), showing the specificity of the reactions.

The original mucin used as a positive control had previously been shown to interact with several of the lectins [17] listed with their abbreviations in Section 4. The results of the present study show that eight of the panel of ten lectins (AAL, LEL, LTL, MAL-I, MPL, RCA-I, SBA, SJA, UEA II and WGA) reacted specifically with a different spectrum of the PLL–oligosaccharide conjugates. LTL stained the entire blot, suggesting that this lectin interacted with the nitrocellulose. SJA reacted with all of the fractions and controls, indicative of non-specific interaction with PLL itself. For the other lectins, there was a wide range of activities with the various HPLC

fractions as summarised in Table 1. MAL-I (Fig. 2(D)) and WGA, which react with sialic acid or GlcNAc and sialic acid respectively, reacted strongly with Fractions 27–37 and with weaker activity against Fractions 25, 26 and 38–43. LEL, which reacts with GlcNAc reacted weakly against Fractions 28, 29 and 33–36 and strongly against Fractions 30–32. SBA and RCA-I, which bind GalNAc and Gal or GalNAc, reacted most strongly with Fractions 30–33 with some weaker activity against Fractions 29, 34–35. MPL, which binds αGalNAc, reacts strongly against Fractions 28–32. The two fucose-specific lectins UEA II and AAL reacted strongly with Fractions 24–32 and AAL also had strongest activity in Fractions 25–29 and weaker activity against Fractions 33–41 (Fig. 2(C)). Complete inhibition of binding of five of the lectins was achieved for AAL, MAL-1, SBA, WGA and RCAI using appropriate monosaccharide controls at the concentrations recommended by the manufacturers.

3. Discussion

The difference in staining patterns between the lectins demonstrates that different oligosaccharides have been released from pigeon mucin by hydrazinolysis and have successfully coupled to PLL, thus allowing binding to the nitrocellulose. Optimisation of conditions at the macro level included both neutral and sialylated oligosaccharides, with the conditions chosen giving maximum yield of both. So far we have not looked at sulfated oligosaccharides or investigated whether sulfate and sialic acid may be partially labile to hydrazinolysis. The structural characterisation of the fractions will be the subject of a subsequent paper, but these were shown by NMR to be free of amino acids and compatible with the oligosaccharide sequences intimated from the lectin binding described above. The NMR also showed a similarity of structural motifs reoccurring in different sized oligosaccharides, consistent with the multiple binding found for the two antisera tested. A test using radiolabelled streptavidin is currently being developed to enable quantification of the antibody responses against the various fractions. A detailed comparison of the IgG and IgG subclass responses of symptomatic and asymptomatic antibody-positive individuals against the oligosaccharides of pigeon intestinal mucin will be the subject of a later paper.

Overall, the results here show that a facile chemical reaction used previously by others at the milligram level [1–4] was developed at the micro-level giving a high degree of sensitivity for detection by lectin overlay using approximately 0.6 μg oligosaccharide (taking on average 30 μg per fraction coupled to PLL in 50 μL buffer with 1 μL of the PLL conjugates then spotted onto the nitrocellulose). The results also demonstrate that the oligosaccharides retain their antigenicity after being released from their original structure and confirm earlier observations that anti-mucin antibodies are carbohydrate specific, as suggested by the retention of antigenicity after

Table 1
Binding of lectins to PLL–oligosaccharide conjugates from PGC-HPLC fractions

Lectin	Reaction with PGC-HPLC fractions														Weak activities
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
AAL	++	++	++	++	++	++	+	+	+	+	+	+	+	+	17–19, 23, 38–51
LEL	−	−	−	−	+	+	++	++	++	+	+	+	+	−	
MAL-1	−	+	+	++	++	++	++	++	++	++	++	++	++	++	38–43
MPL	−	−	−	−	−	−	−	−	−	++	++	++	++	−	
RCA-1	−	−	−	−	++	++	++	++	++	++	−	−	−	−	
SBA	−	−	−	−	++	++	++	++	++	++	+	−	−	−	
UEAII	++	++	++	++	++	++	++	++	++	−	−	−	−	−	
WGA	−	+	+	++	++	++	++	++	++	++	++	++	++	++	38–43

exhaustive papain digestion [18]. The status of oligosaccharide synthesis of mucins has advanced considerably over the last few years to the level of several different core regions and glycopeptides [19] being available, but in order to have longer, antigenically active molecules for immunological and functional studies, we need to resort to analysis methods such as that described here. Although not optimised for different oligosaccharides, it gives an early identification of structure–activity relationships not possible from fully purified, rather that fractionated, material.

4. Experimental

Materials.—PLL hydrobromide (mol wt 8 kD) was from Sigma, Poole, UK. Pigeon intestinal mucin was obtained from the intestines of freshly killed pigeons by CsCl density gradient centrifugation as described previously [12]. Sera were collected from two pigeon fanciers with the classic late (4–8 h) respiratory and systemic symptoms associated with PFL. These were shown to have precipitating antibodies to pigeon faeces and pigeon serum, detected as in Ref. [12] and IgG titres of 367,000 and 350,000, respectively, in an ELISA against mucin. Sera were also collected from two laboratory workers who had no contact with pigeons or other species of birds. HPLC was carried out using a Gilson System consisting of two model 302 pumps, a model ST1 dynamic mixer and a model 802C anomeric module with vacuum degasser. The detector was a Waters 486 Tunable Absorbance Detector set at 210 nm.

Hydrazinolysis.—Pigeon intestinal mucin (3 mL) at a concentration of 3 mg/mL carbohydrate as measured by a PAS method [20] was lyophilised overnight and stored over phosphorus pentoxide for 24 h before hydrazinolysis was carried out using a Glycorelease N- and O-glycan recovery kit (Oxford GlycoSciences, Abingdon, UK) as per the manufacturer's recommendations. O-Glycans were released by incubation with 5 mL hydrazine in a sealed vial placed in a heating block at 60 °C for 4 h. The reaction vessel was cooled to room temperature (rt) and excess hydrazine was removed by centrifugal evaporation under reduced pressure. For safety, the evaporator was vented to a fume cupboard and kept on overnight after the sample had been dried. The released oligosaccharides were N-reacetylated by addition of 0.5 mL ice-cold acetylation buffer and 50 μL of acetylating reagent (as supplied) with incubation for 20 min at 4 °C and then 30 min at rt. The oligosaccharides were then de-salted on a cation-exchange resin and dried using a centrifugal evaporator. The dried samples were dissolved in a minimal amount of pure water and the released glycans were purified from peptide material by ascending paper chromatography using high purity 16:4:1 butanol–ethanol–water. The oligosaccharides were eluted at, and a few centimetres in front of, the origin using water and then filtered through a 0.2 μm PTFE filter, evaporated to dryness and finally dissolved in 100 μL water and stored at −80 °C.

RP-HPLC on PGC.—One fifth of the released oligosaccharides were lyophilised and redissolved in 0.1% TFA for loading onto a porous graphitised carbon (PGC) column (Hypersil Ltd, Runcorn, Cheshire, UK) and fractionated using a gradient of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) at a flow rate of 1 mL/min with 0–60% B over 60 min, 60% B for 10 min and 60–0% B over 2 min. 70 Fractions of 1 mL were collected and evaporated to dryness in a gyrovap. They were taken up in 60 and 10 μL portions pipetted into a microtitre plate for assay of amount of hexose present by addition of 50 μL of 2% aqueous phenol and 200 μL concd H_2SO_4. The results were read in a microtitre plate reader at 490 nm and calibrated against galactose as standard. The hexose assay was also used to quantitate the amount of material after coupling to PLL and dialysis (Slide-A-Lyzer, Pierce and Warriner, Chester, UK) during optimisation of the reaction.

Coupling of oligosaccharides to PLL.—The optimisation of the reaction using lactose and sialyl lactose showed that maximum yields were obtained under the following conditions: 100 μg oligosaccharide (approx. 100 nmol) and 1 nmol of PLL (8000 mol wt; 8 μg) in 110

μL phosphate buffer pH 7.4 incubated at 40 °C for 1 h, followed by addition of 10 μL of cyanoborohydride solution (300 nmol, 20 μg) in phosphate buffer and left overnight. This was modified for convenience to the following reaction conditions: 50 μL of 0.092 M sodium phosphate pH 7.4 containing 32 μg/mL of PLL (200 pmol in 50 μL) was added to each oligosaccharide fraction recovered from RP-HPLC on PGC (assuming 20 μg oligosaccharide in each tube) and incubated at 37 °C for 2 h. Sodium cyanoborohydride (60 nmol) (4 μg in 5 μL water) was added to each tube and incubated for 16 h at rt. Aliquots (5 μL) of the oligosaccharide–PLL conjugates were stored at −80 °C until further use, avoiding repeated freeze–thaw cycles.

Lectin dot blots.—The polyvalent oligosaccharide–PLL conjugates were diluted 1:2 in PBS and 2 × 1 μL of each sample was spotted, 1 cm apart, onto 0.2 μM immobilon nitrocellulose membrane (NCM, Millipore Corp, Bedford, USA), taking care to allow the samples to dry completely before the second application. Pigeon intestinal mucin (2 × 1 μL, 1 μL at 100 μg/mL as measured by PAS), unreacted PLL and PBS were spotted on the NCM as positive and negative controls, respectively. The samples were left at rt to dry completely and the blots were washed twice, for 5 min each wash, in PBS containing 0.1% Tween 20 (PBS–T). This and all subsequent incubations were carried out on a rocking table at rt. After washing, the dot blots were blocked in PBS–T containing 5% bovine serum albumin (PBS–T–BSA) for 1 h and then washed a further four times in PBS–T. The following biotinylated lectins (Vector Labs, Peterborough, UK) were diluted to 5 μg/mL in PBS–T–BSA, added to each blot and incubated for 2 h: *Aleuria aurantia* (AAL), *Lotus tetragonolobus* (LTL), *Lycopersicon esculentum* (LEL), *Maackia amurensis* (MAL-1), *Maclura pomifera* (MPL), soybean agglutinin (SBA), *Sophora japonica* (SJA), *Ricinus communis* agglutinin (RCA-1), *Ulex europeus* II (UEAII), and wheat germ agglutinin (WGA). The dot blots were washed as previously and incubated in ExtrAvidin peroxidase conjugate (Sigma), diluted 1/500 in PBS–T–BSA, for 45 min. Finally, the dot blots were washed three times in PBS–T and twice in PBS and then incubated in the developing solution [60 mg 4-chloro-1-naphthol (Sigma) dissolved in 20 mL cold methanol, 80 mL cold PBS and 60 μL 6% hydrogen peroxide] until spots appeared on the dot blot. The reaction was stopped by washing the blots extensively (at least 20 changes) in distilled water

Antibody dot blots.—IgG antibodies were detected essentially as above with the following modifications. The polyvalent oligosaccharide–PLL conjugates were diluted 1/5 in PBS and 1 μL of each sample was spotted onto the NCM. After blocking and washing, the sera from both pigeon fanciers and normals were diluted 1/5000 in PBS–T–BSA and added to each blot and incubated for 2 h. The dot blots were washed in PBS–T and then incubated with a 1/500 dilution of biotin conjugated rabbit anti-human IgG (DAKO Ltd, Denmark) in PBS–T–BSA for 90 min, washed again and incubated in ExtrAvidin peroxidase conjugate as previously. Finally, the blots were incubated in the developing solution until spots appeared and the reaction was stopped by washing extensively in distilled water. From ELISA experiments on whole mucin, it was shown that preincubation with lectin will not inhibit antibody binding as the patients antibodies have higher affinity.

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