Analysis of IgA1 *O*-Glycans in IgA Nephropathy by Fluorophore-Assisted Carbohydrate Electrophoresis

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Abstract. Abnormal *O*-glycosylation of IgA1 may contribute to pathogenic mechanisms in IgA nephropathy (IgAN). Observations of altered lectin binding to IgA1 in IgAN suggest that the *O*-glycan chains may be undergalactosylated, but precise structural definition of the defect has proved technically difficult, and it remains unconfirmed. This is the first study using fluorophore-assisted carbohydrate electrophoresis (FACE) to analyze IgA1 *O*-glycans in IgAN and controls. IgA1 was purified from serum, and the intact *O*-glycans were released by hydrazinolysis at 60°C. After re-*N*-acetylation, the glycans were fluorophore-labeled and separated by polyacrylamide gel electrophoresis. Sequential exoglycosidase digestions of IgA1 allowed identification of the different *O*-glycan bands on FACE gels, and their relative frequencies in IgA1 samples were measured by ultraviolet densitometry. Lectin binding of the IgA1 samples was also measured. In some patients with IgAN, FACE analysis demonstrated a significant increase in the percentage of IgA1 *O*-glycan chains consisting of single *N*-acetyl galactosamine (GalNAc) units rather than the more usual galactosylated and sialylated forms. This finding was confirmed using both desialylated IgA1 and enzymatically released *O*-glycans. Good correlation was also found between *O*-glycan agalactosylation on FACE analysis and IgA1 lectin binding in IgAN, supporting the value of lectins as tools for detection of this abnormality. This is the first study in which all of the predicted *O*-glycan forms of IgA1 have been analyzed simultaneously, and demonstrates that in IgAN, the IgA1 *O*-glycan chains are truncated, with increased terminal GalNAc. This abnormality has the potential to significantly affect IgA1 behavior and handling with pathogenic consequences in IgAN.

IgA nephropathy (IgAN) is characterized by deposition of IgA1 molecules in the glomerular mesangium. The mechanisms involved in mesangial IgA1 deposition, and the initiation of inflammatory glomerular damage that ensues in a significant proportion of patients, remain obscure. A variety of modest abnormalities of the IgA immune system are recognized in IgAN (1), but none of these can satisfactorily account for mesangial deposition. The deposited IgA1 appears to be polyspecific, displaying no apparent antigen restriction. For this reason, physicochemical abnormalities of IgA1, which may promote deposition in a nonimmunologic manner, have been sought, and we and others have described abnormal *O*-glycosylation of serum IgA1 in IgAN (2–8).

Glycosylation of extracellular proteins occurs in two major forms. Carbohydrate moieties may be *N*-linked to asparagine residues or *O*-linked to serine or threonine residues. Complex *N*-glycan chains are a common feature of serum proteins including all Ig isotypes. However, although several membrane-bound proteins are heavily *O*-glycosylated, this is a feature displayed by very few serum proteins.

In addition to its *N*-glycosylation, human IgA1 has a series of *O*-glycans linked to serine or threonine residues in the hinge region of the molecule, a short repeating sequence of amino acids lying between the CH1 and CH2 domains of the α1 heavy chain (Figure 1) (9,10). Each *O*-glycan is based on a core *N*-acetyl galactosamine (GalNAc) unit in *O*-linkage with the amino acid backbone. The chain may be extended by the sequential addition of galactose (Gal) in β1,3 linkage with GalNAc and with one or two sialic units in α2,3 linkage with Gal or α2,6 linkage with GalNAc (11,12). Thus, each *O*-glycan may consist of one of four different forms, A through D, illustrated in Figure 2. These *O*-glycans are identical to those displayed by membrane-bound proteins such as mucins. In this context, the unsialylated Galβ1,3GalNAc moiety (form B) is often termed the Thomsen-Friedenreich, or T, antigen, while the agalactosyl GalNAc moiety (form A) is referred to as the Tn antigen (13).

Normal serum IgA1 consists of a mixture of *O*-glycoforms due to the heterogeneity afforded both by varied site occupancy (up to five *O*-glycans per α1 chain have been described ([10,14])) and by the occurrence of a mixture of the four chain types described above. A number of experimental approaches to the study of IgA1 *O*-glycosylation have been used in IgAN: (*1*) lectin binding to the *O*-glycans *in situ* on IgA1; (*2*) mass spectroscopy of the isolated IgA1 hinge region peptide complete with its *O*-glycans; and (*3*) chromatographic separation of the released *O*-glycans, both in their intact form and as free monosaccharides. All of these techniques have shown abnormal IgA1 *O*-glycoform patterns in IgAN, but as yet none has been able fully to elucidate the nature of the abnormality.

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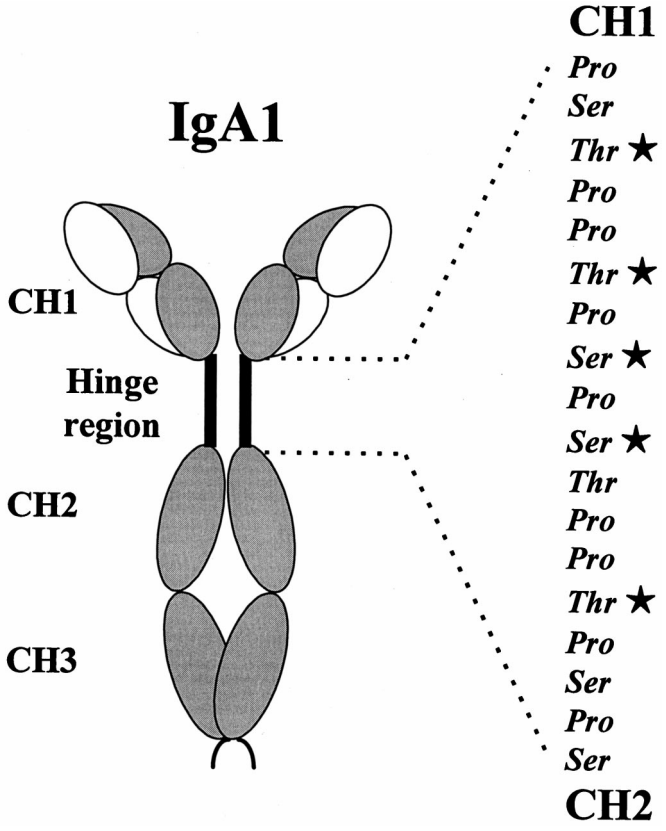

Figure 1. Position of IgA1 hinge region *O*-glycosylation sites. On the left is the human IgA1 molecule, showing the location of the hinge region between the CH1 and CH2 domains. On the right is the amino acid sequence of the hinge region. All of the serine and threonine residues are potential *O*-glycosylation sites, but in a recent study (10), only those marked with a star were found to be occupied.

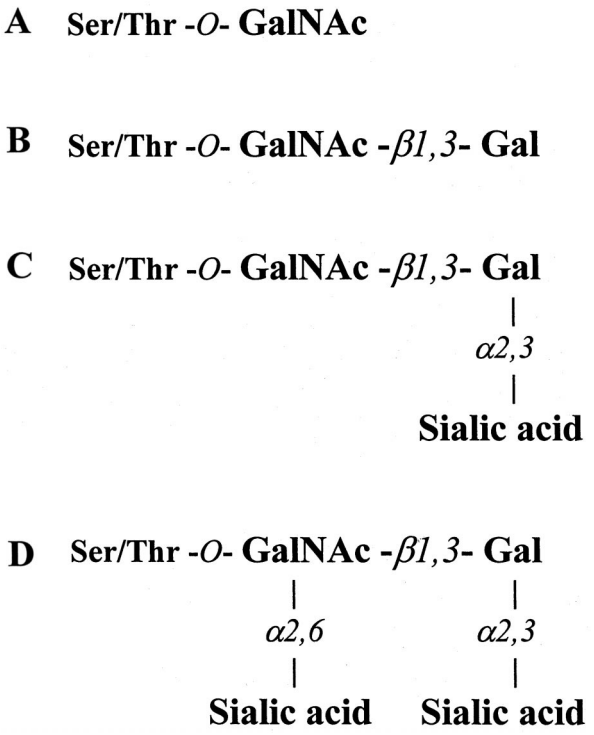

Figure 2. The four major *O*-glycan forms of human IgA1. The IgA1 *O*-glycans are all based on a core *N*-acetyl galactosamine (GalNAc) unit in *O*-linkage with serine or threonine. This may occur alone (form A) or may be extended with β1,3-linked Gal (form B). Further extension with sialic acid in α2,3 linkage with Gal can then occur (form C), and finally, addition of sialic acid in α2,6-linkage with GalNAc makes form D.

Several studies have demonstrated altered binding of various *O*-glycan-specific lectins to IgA1 (2–7). In IgAN, IgA1 shows increased binding to GalNAc-specific lectins such as *Vicia villosa* (2,7) and decreased binding to jacalin (3–5), a lectin with highest affinity for the galactosylated glycan forms B through D. Although lectins cannot give precise structural information about the glycans they bind to, these observations suggest that there may be an increased frequency of *O*-glycans consisting of GalNAc alone (form A). This supposition has been supported to some extent by mass spectroscopy of desialylated IgA1 hinge region peptides (8) and by gas-liquid chromatography of mixed *N*- and *O*-linked monosaccharides released from IgA1 (5). However, full characterization of this glycosylation defect remains elusive. Analysis of the sialylated glycans has proved particularly difficult, but is pivotal since sialic acid is highly charged and labile, and likely to be of functional importance. The lectin binding alterations alluded to above could conceivably be a consequence of reduced *O*-sialylation, allowing lectins better access to the core glycan units proximal to the protein backbone. Unfortunately, although mass spectroscopy has provided some useful information about the degree of hinge region glycan site occupancy and about the desialylated glycan chains (8,14), this technique has proved less satisfactory for the analysis of the sialylated moieties. Reverse-phase HPLC has been used to analyze the relative frequencies of the larger glycan chains, but the single GalNAc unit could not be detected (15,16). As yet, no technique has provided simultaneous analysis of all four intact IgA1 *O*-glycan types.

Fluorophore-assisted carbohydrate electrophoresis (FACE) is a method of glycan analysis (17–19) in which complete glycan chains are first released intact from a glycoprotein by chemical or enzymatic means. The glycans are then stoichiometrically labeled with a fluorophore and separated by polyacrylamide gel electrophoresis. The resultant bands, representing each of the four *O*-glycan forms A through D, can then be visualized under ultraviolet (UV) light and quantified by densitometry. Although there are a number of published studies using FACE for *N*-glycan analysis, this technique has been less often applied to the study of *O*-glycosylation.

We report here the first study using FACE to analyze the *O*-glycans of IgA1. In IgA1 from IgAN, we found an increased frequency of *O*-glycans consisting of GalNAc alone, rather than the galactosylated and sialylated forms. We also demonstrated that in IgAN, the frequency of single GalNAc units correlated well with both *Vicia villosa* lectin and anti-Tn antibody binding to the same IgA1 sample. This observation lends credence to the use of lectins and anti-Tn as tools to detect *O*-glycosylation changes in IgA1 in this disease.

Materials and Methods

Materials

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (Dorset, United Kingdom). Anti-C1 inhibitor antibodies were obtained from The Binding Site (Birmingham, United Kingdom), and all other antibodies and OPD enzyme-linked immunosorbent assay (ELISA) substrate were from Dako Ltd. (Ely, United Kingdom). Exo- and endoglycosidases were from Oxford GlycoSciences (Oxford, United Kingdom), and Glyko FACE reagents were obtained from Dextra Laboratories (Reading, United Kingdom).

Subjects

Serum was obtained with informed consent from 22 patients with biopsy-proven IgAN (14 male; median age 43.5; range, 22 to 65 yr) attending the Nephrology Clinic at Leicester General Hospital, and 23 healthy control subjects (15 male; median age 43.0; range, 20 to 73 yr). No patient had macroscopic hematuria or renal insufficiency at the time of the study, and the control subjects had no evidence of renal or systemic disease. The study was approved by the Research Ethics Committee of Leicestershire Health Authority.

Preparation of Serum IgA1

Serum IgA1 was purified by affinity chromatography on jacalin agarose (Vector Laboratories, Peterborough, United Kingdom), as follows. Ten milliliters of serum was precipitated with 10 ml of 45% ammonium sulfate in phosphate-buffered saline (PBS). The supernatant was discarded and the precipitate was redissolved in 10 ml of 0.175 M Tris-HCl buffer, pH 7.5. The solution was mixed with 5 ml of packed agarose-bound jacalin and stirred gently for 1 h at room temperature. The jacalin-agarose was washed extensively in Tris-HCl buffer to remove unbound proteins, and the IgA1 was eluted from the jacalin by stirring in 10 ml of 1 M galactose in Tris-HCl for 1 h at room temperature. The IgA1 was then dialyzed against PBS and stored in aliquots at −20°C until needed. The IgA1 and C1 inhibitor (C1inh) concentrations of the samples were measured by ELISA, and the purity of the preparations was demonstrated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. A larger pool of IgA1 from normal sera was also prepared for the purpose of developing the techniques described below. When required for subsequent techniques, 1-mg aliquots of IgA1 were extensively dialyzed against ultrapure water and dried by centrifugal evaporation in a Savant SpeedVac (CP Instrument Co. Ltd, Bishops Stortford, United Kingdom) at ambient temperature immediately before use.

Exoglycosidase Treatment of IgA1

Total desialylation of IgA1 was carried out using a recombinant preparation of *Clostridium perfringens* sialidase, which has no effective preference for α2,3- or α2,6-linked sialic acid (20). Dry IgA1 samples (1 mg) were saponified in 50 mM NaOH at 4°C for 30 min, followed by neutralization with an equal volume of 50 mM HCl. Desialylation was carried out overnight at 37°C in 0.05 M sodium acetate, pH 5, using 0.2 U sialidase/1 mg IgA1.

α2,3 linkage-specific desialylation of IgA1 was carried out with the sialidase from Newcastle disease virus (Hitchner B1 strain), which is reported to display absolute specificity for the α2,3 linkage (20). One milligram of IgA1 in 100 μl of 50 mM sodium acetate, pH 5.5, was digested with 20 mU of enzyme overnight at 37°C.

Degalactosylated IgA1 was prepared by digestion with β-galactosidase from bovine testes. After total desialylation and dialysis, 1 mg of IgA1 was incubated overnight at 37°C in 40 μl of 100 mM sodium citrate/phosphate buffer, pH 4, with 30 mU of enzyme.

De-N-Glycosylation

In some instances, the endoglycosidase peptide-*N*-glycosidase F (PNGaseF) from *Flavobacterium meningosepticum* was used to release the *N*-glycans from IgA1 before subsequent *O*-glycan release and FACE analysis. One milligram of IgA1 was saponified and desialylated with *Clostridium perfringens* sialidase as described above. To expose the *N*-glycosylation sites, the protein was denatured by boiling for 2 min in 50 μl of PNGaseF incubation buffer (20 mM sodium phosphate, pH 7.5, 50 mM ethylenediaminetetra-acetic acid) containing 0.5% SDS and 5% β-mercaptoethanol. Ten microliters of 5% Nonidet P-40 was added and de-*N*-glycosylation was carried out with 6 U of PNGaseF, incubating overnight at 37°C. The samples were dialyzed to remove the freed *N*-glycans, and *O*-glycan release was carried out as described below.

Release of O-Glycans

Intact *O*-glycans were released from 1-mg aliquots of native, saponified, desialylated, degalactosylated, and de-*N*-glycosylated IgA1 by hydrazinolysis at 60°C (21). The samples were extensively dialyzed against ultrapure water, dried by centrifugal evaporation, and further dried overnight under vacuum in the presence of P_2O_5. Forty microliters of anhydrous hydrazine was added to each sample, overlaid with N_2, and incubated at 60°C for 3 h. The samples were then dried by centrifugal evaporation.

In addition, enzymatic release of *O*-glycans was carried out on 12 samples (six IgAN and six controls) by sequential digestions with endo-α-*N*-acetyl galactosaminidase (*O*-glycanase) from *Streptococcus pneumoniae*, which cleaves the disaccharide Galβ1,3GalNAc from serine or threonine residues (22), and α-*N*-acetyl galactosaminidase (GalNAcase) from chicken liver, which cleaves the single *O*-linked GalNAc units from the protein (23). After total desialylation, dialysis, and drying, 1-mg aliquots of IgA1 were redissolved in 100 μl of incubation buffer (100 mM sodium citrate/phosphate, pH 6, with 100 μg/ml bovine serum albumin and 0.02% sodium azide) containing 0.5% SDS. The IgA1 was boiled for 5 min to denature the protein, and a further 400-μl incubation buffer was added, containing 6.25% Nonidet P-40. Release of Galβ1,3GalNAc (form B) was carried out by 24-h incubation at 37°C with 1 mU of *O*-glycanase per sample. The pH was then adjusted to 4 with 2 M citric acid, 3 mU GalNAcase added, and the samples were incubated at 37°C for a further 24 h for release of the single GalNAc moieties (form A). The samples were passed through 2-ml columns containing Dowex 50W-X8 ion exchange resin (H^+ form), eluting the released glycans with 5 ml of ultrapure water. The glycan samples were dried by centrifugal evaporation before fluorophore labeling. To assess the efficacy of glycan release by this method, a test aliquot of normal pooled IgA1 was sequentially digested with the endoglycosidases, dialyzed to remove released *O*-glycans, and then subjected to the hydrazinolysis procedure described above.

Re-N-Acetylation

After hydrazinolysis of IgA1, the freed glycans were re-*N*-acetylated by two 30-min incubations at room temperature with 125 μl of 0.1 M $NaHCO_3$ and 12.5 μl of acetic anhydride. The samples were then passed through 2-ml Dowex columns as described above, and dried by centrifugal evaporation.

Fluorophore Labeling

The *O*-glycans were labeled with a Glyko preparation of the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS). One vial of ANTS was reconstituted in 125 μl of 15% acetic acid and 5 μl was added to each dry *O*-glycan sample. When the samples had dissolved, 5 μl of 1 M sodium cyanoborohydride in DMSO was added, and labeling was carried out overnight at 37°C. The samples were dried by centrifugal evaporation for approximately 20 min until a gel consistency was reached, diluted in 10 μl of water, and stored at −80°C.

Fluorophore-Assisted Carbohydrate Electrophoresis

For separation of *O*-glycans by polyacrylamide gel electrophoresis, a 4-μl sample was diluted in an equal volume of 40% glycerol in water, and 8 μl was loaded into the wells of Glyko FACE *O*-linked profiling gels. Electrophoresis was carried out using a Glyko FACE gel box and Glyko FACE running buffer, running two gels at a time at 40 mA for 1 h at 10 to 20°C. Immediately after electrophoresis, the gels were viewed under UV light, and the band densities were analyzed using a UV gel camera with the Molecular Analyst densitometry software package (BioRad, Hemel Hempstead, United Kingdom).

Identification of Bands

To identify the band positions of the different forms of *O*-glycans on FACE gels, replicate aliquots of a normal pooled IgA1 sample were saponified, desialylated, and degalactosylated, and prepared for FACE as detailed above. In addition, the free sugars *N*-acetyl neuraminic acid (sialic acid), *N*-acetyl glucosamine (GlcNAc), Gal, GalNAc, and Galβ1,3GalNAc (a kind gift from Dr. Chris Lawson, Dextra Laboratories, Reading, United Kingdom) were fluorophore-labeled as described and run on FACE gels as markers. In this way, the four basic *O*-glycans of IgA1 (forms A through D) were identified. Samples of IgG and IgA2 (which lack *O*-glycosylation sites), and of fetuin and bovine submaxillary mucin (which are both heavily *O*-glycosylated) were treated in exactly the same way as IgA1 to confirm that the bands obtained did indeed represent *O*-glycans. We also attempted to determine the identity of an unknown sialylated band by digestion of IgA1 with the α2,3-linked sialic acid-specific sialidase from the Newcastle disease virus. Finally, the band patterns obtained from IgA1 with and without pretreatment with PNGaseF were compared to establish whether any of the bands were derived from *N*-glycans.

FACE Analysis of IgA1 in IgAN Patients and Control Subjects

One-milligram aliquots of native (undigested) IgA1 from 20 patients with IgAN and 20 control subjects were prepared for FACE by hydrazinolysis, re-*N*-acetylation, and fluorophore labeling as described. In addition, IgA1 from eight patients and eight control subjects was totally desialylated with *Clostridium perfringens* sialidase, before *O*-glycan release by hydrazinolysis as before. Finally, the *O*-glycans were enzymatically released from the desialylated IgA1 of six patients and six control subjects, fluorophore-labeled, and analyzed by FACE.

Vicia Villosa Lectin Binding and Anti-Tn Assays

Vicia villosa (VV) lectin binding of the IgA1 samples was measured in an ELISA-type system as described previously (2). In addition, the binding of a monoclonal antibody against the Tn antigen (GalNAc; form A) to the IgA1 samples was measured in a similar manner. Briefly, purified IgA1 samples were applied to the wells of duplicate 96-well immunoplates at equivalent, plate-saturating concentrations (50 μg/ml), and incubated overnight at 4°C. After washing 4 times with PBS/0.3 M NaCl/0.1% Tween 20, the plates were blocked with 5 mg/ml oxidized glutathione in PBS for 1 h at room temperature and washed again. Biotinylated VV lectin (1 μg/ml) or anti-Tn (1:500) in PBS were applied to the duplicate plates and incubated for 2 h at room temperature. After further washing, VV or anti-Tn binding was detected with horseradish peroxidase-conjugated avidin (Vector Laboratories) at 2.5 μg/ml in PBS or horseradish peroxidase-conjugated anti-mouse Ig antibody at 1:500 in PBS, respectively, for 2 h at room temperature, and the plates were washed again. The reaction was developed with OPD/H_2O_2 substrate, stopped with 1 M H_2SO_4, and the results were read as absorbance at 492 nm and expressed in arbitrary units (AU)/ml.

Statistical Analyses

FACE analysis results were obtained by measuring the densities of the bands of interest in each lane on the gels, and expressing them as percentages of the lane total to allow for variable sample loading. Unpaired *t* tests were used to compare the percentages represented by each band on FACE gels, and to compare the lectin and anti-Tn binding results between the patient and control groups. Linear regression analysis was used to investigate the relationships between the results obtained from *O*-glycan analysis by the different methods.

Results

Purification of Serum IgA1

Approximately 4 mg of IgA1 was obtained from each 10-ml serum sample by affinity chromatography on jacalin agarose. The IgA1 samples were shown to be >90% pure by SDS-polyacrylamide gel electrophoresis. C1inh is the only other major serum protein with *O*-linked glycosylation. This protein was present at less than 5% of the IgA1 concentration in the samples, and since C1inh carries a single *O*-glycan—while IgA1 may have up to 10 *O*-glycans per monomer—we estimated that at least 99% of the *O*-glycans in the samples would be derived from IgA1.

FACE Analysis of IgA1 Glycans Released by Hydrazinolysis at 60°C

Release of *O*-glycans from 1 mg of IgA1 provided sufficient material for two to three FACE analyses. When examined under UV light, six major bands, termed bands 1 to 6, were observed in lanes containing glycans derived from IgA1 by hydrazinolysis at 60°C (Figure 3, lane a). Similar treatment of IgA2 and IgG, which do not carry *O*-glycans, gave the lower two bands (bands 5 and 6) only (Figure 3, lanes b and c, respectively), indicating that although bands 1 to 4 were likely to represent *O*-glycan chains, bands 5 and 6 may also be derived from *N*-linked glycans. Fetuin and bovine submaxillary mucin, two heavily *O*-glycosylated proteins, gave similar band patterns to IgA1.

Identification of O-Glycan Forms A through D on FACE Gels

The positions of the GalNAc and Galβ1,3GalNAc moieties (glycan forms A and B) were determined by comparison with

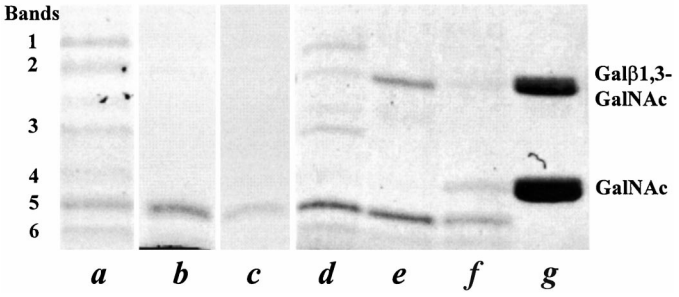

Figure 3. Identification of *O*-glycans A through D on fluorophore-assisted carbohydrate electrophoresis (FACE) gels. Lane a, *O*-glycans released from IgA1 by hydrazinolysis at 60°C gave six major bands, labeled 1 to 6. Lanes b and c, IgA2 (b) and IgG (c) subjected to the same treatment gave faint bands 5 and 6 only. Lanes d through f, Sequential digestion of IgA1 with exoglycosidases to identify the bands. Lane d, Native IgA1. Lane e, IgA1 digested with sialidase from *Clostridium perfringens* demonstrates that bands 1 and 3 are sialylated, since they are absent after desialylation. Lane f, IgA1 further digested with β-galactosidase. Band 2, diminished after degalactosylation, is the galactosylated form of band 4, which is correspondingly increased. Lane g contains free sugars Galβ1,3GalNAc (top, band 2) and GalNAc (bottom, band 4).

lanes containing these free sugars (Figure 3, lane g). Thus, bands 2 and 4 were identified as glycan forms B and A, respectively. Comparison of lanes containing native and desialylated IgA1 samples (Figure 3, lanes d and e) indicated the positions of two major sialylated bands (bands 1 and 3, assumed to be glycan forms C and D), and also confirmed the identity of the Galβ1,3GalNAc band (band 2, glycan form B), which showed increased intensity in the desialylated IgA1 when compared with the *O*-glycans of the same IgA1 sample in its native form. The identity of this band, and that of the GalNAc band 4, was further confirmed by degalactosylation of desialylated IgA1 (Figure 3, lane f); in this case, the Galβ1,3GalNAc band 2 was diminished and the intensity of the GalNAc band 4 increased correspondingly.

Further Analysis of the Sialylated O-Glycan Bands on FACE Gels

Two sialylated *O*-glycan bands (bands 1 and 3) were identified by total desialylation of IgA1. The mobility of glycan chains in FACE gels is determined by a combination of their size and charge. The sialylated chains, although larger than the unsialylated neutral glycans A and B, are negatively charged and therefore their positions in the gel cannot be predicted by size alone. From comparison of our gels with published data (19), we deduced that band 1 is most likely to be the disialylated *O*-glycan D. Treatment of IgA1 with Newcastle disease virus sialidase resulted in the loss of both sialylated bands, and increased the intensity of band 2. Therefore, band 3 probably represents the α2,3-monosialylated *O*-glycan C.

Identification of Bands 5 and 6 on FACE Gels

Bands 5 and 6 appeared consistently in material derived from glycoproteins with and without *O*-glycosylation. Band 5 ran in the same position as the free sugar galactose. Band 6 did not migrate in the same position as any of the free sugars available, and its identity remained obscure. These bands were observed in hydrazinolyzed IgA1 preparations after prior enzymatic removal of *N*-glycans, and in IgA1 *O*-glycan preparations released by endoglycosidases rather than by hydrazinolysis. Therefore, it would appear that these two bands are derived from a mixture of *N*- and *O*-glycans, and although they may represent some glycan degradation during processing, they are not a result of hydrazinolysis alone.

Analysis of Native IgA1 O-Glycans from IgAN Patients and Control Subjects Released by Hydrazinolysis at 60°C

The IgA1 *O*-glycans were released from the IgA1 of 20 patients and 20 control subjects by hydrazinolysis and analyzed by FACE. The densities of the four *O*-glycan bands 1 to 4 (glycan forms A through D) were measured and expressed as relative percentages of the total for each lane, to allow for

Table 1. FACE analysis of IgA1 *O*-glycans in IgAN and control subjects[a]

O-Glycan Form	IgAN	Control	*P* Value
Native IgA1	$n = 20$	$n = 20$	
%GalNAc (A) (band 4)	22 (2)	13 (1)	<0.001
%Galβ1,3GalNAc (B) (band 2)	26 (3)	30 (3)	NS
%sialylated band 3 (?monosialyl-Galβ1,3GalNAc [C])	27 (2)	27 (3)	NS
%sialylated band 1 (?disialyl-Galβ1,3GalNAc [D])	25 (2)	30 (3)	NS
sialyl:asialyl ratio (bands 1 + 3: band 2)	0.54 (0.07)	0.62 (0.11)	NS
galactosyl:agalactosyl ratio (bands 1 + 2 + 3: band 4)	0.3 (0.03)	0.15 (0.01)	<0.001
Desialylated IgA1 (released by hydrazinolysis)	$n = 8$	$n = 8$	
%GalNAc (A)	48 (2)	40 (2)	0.017
Desialylated IgA1 (enzymatic release)	$n = 6$	$n = 6$	
%GalNAc (A)	53 (4)	39 (2)	0.026

[a] Results are given as mean (±SEM). IgAN, IgA nephropathy.

variable lane loading. The results are shown in Table 1. There was no difference between patients and control subjects in the percentage of total represented by bands 1 to 3 (glycan forms B through D). However, in IgAN, the percentage of total represented by band 4 (GalNAc; glycan form A) was significantly higher than that of control subjects, as illustrated in Figures 4 and 5A. The ratio of sialylated:unsialylated Galβ1,3GalNAc chains (bands 1 + 3: band 2) did not differ between patients and control subjects, but the ratio of galactosylated:ungalactosylated moieties (bands 1 + 2 + 3: band 4) was significantly raised in IgAN (Table 1).

Bands 5 (Gal) and 6 were obtained from all IgA1 samples. There was no difference in the densities of these bands between IgAN and control subjects (Figure 5B).

Analysis of O-Glycans Released from Desialylated IgA1 in IgAN Patients and Control Subjects

The *O*-glycans were released by hydrazinolysis from the *Clostridium perfringens* desialylated IgA1 of eight patients and eight control subjects and analyzed as above. Again, the percentage of *O*-glycans consisting of GalNAc alone was significantly higher in patients than control subjects (Table 1).

Analysis of Enzymatically Released IgA1 O-Glycans in IgAN Patients and Control Subjects

The *O*-glycans were released from the IgA1 of six patients and six control subjects by sequential digestion with two endoglycosidases. Because these enzymes do not cleave sialylated moieties, it was necessary to use totally desialylated IgA1 for this experiment. To confirm the efficacy of glycan release by this method, a test IgA1 sample was treated with the endoglycosidases, dialyzed to remove the freed sugars, and then subjected to the hydrazinolysis procedure. No glycan bands were seen when this sample was run on FACE. By this method also, patients with IgAN had a higher percentage of glycans consisting of GalNAc alone than did control subjects (Table 1).

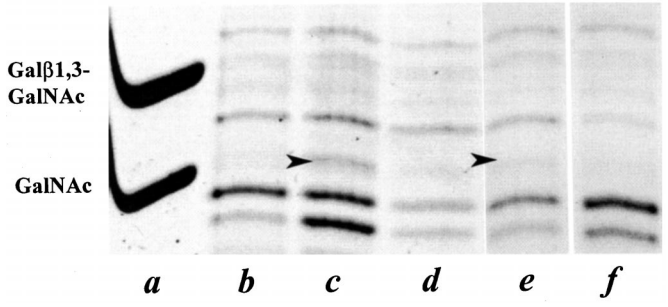

Figure 4. FACE analysis of *O*-glycans of IgA1 in IgAN and controls. Lane a contains free sugars Galβ1,3GalNAc (top) and GalNAc (bottom) as markers. Lanes b through f contain *O*-glycans released from native IgA1 by hydrazinolysis at 60°C. Lanes b, d, and f, controls; Lanes c and e, IgAN. In IgAN, there is a marked increase in the intensity of the GalNAc band (marked by arrowhead).

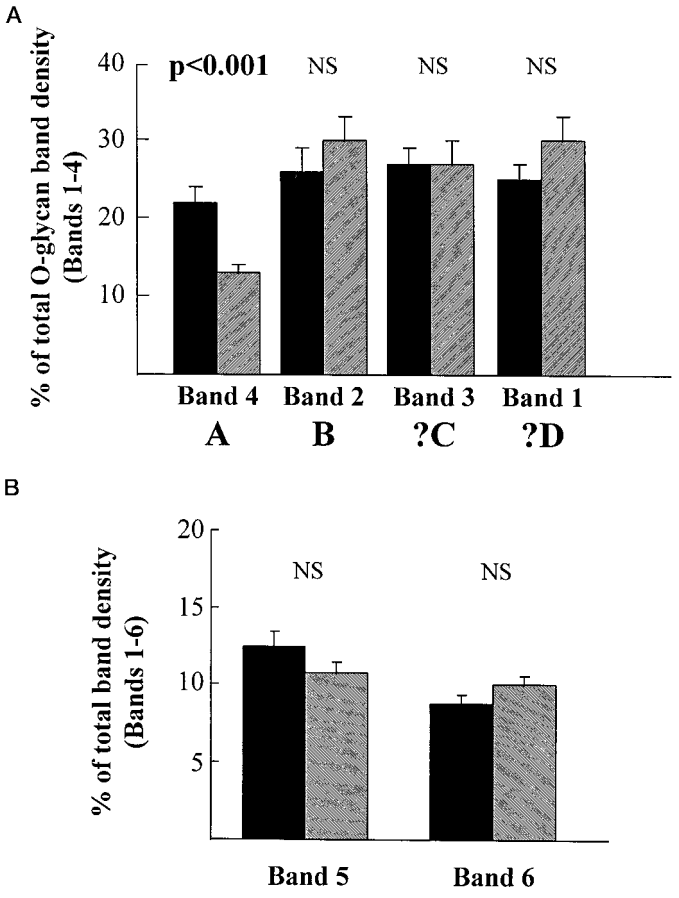

Figure 5. Relative frequencies of bands 1 to 6 in IgAN and controls. Native IgA1 from 20 IgAN and 20 controls; glycan release by hydrazinolysis at 60°C. Bars show mean ± SEM; Black bars, IgAN; grey bars, control. (A) *O*-glycan forms A through D expressed as percentage of bands 1 through 4 total. In IgAN, the percentage of glycan A (GalNAc) is significantly higher than in controls ($P < 0.001$), but the percentages of glycans B through D do not differ between the groups. (B) Bands 5 and 6 expressed as percentage of total bands 1 through 6. These two bands do not differ between the IgAN and control groups.

VV Lectin and Anti-Tn Binding of IgA1 in IgAN and Control Samples

The VV lectin and anti-Tn binding of the 20 IgAN and 20 control IgA1 samples used for FACE analysis of native IgA1 above were measured by our ELISA-type method. As shown in Figure 6, IgA1 from patients with IgAN showed higher lectin binding than controls (IgAN 1.66 ± 0.07, control 1.40 ± 0.05 AU/ml, $P = 0.004$). Similarly, the binding of the anti-Tn monoclonal antibody to IgA1 was higher in IgAN than controls (IgAN 1.64 ± 0.04, control 1.48 ± 0.03 AU/ml, $P = 0.006$). Not surprisingly, since VV lectin and anti-Tn antibody recognize the same target, namely GalNAc, there was a good correlation between the results obtained from these two assays ($r = 0.6$, $P < 0.001$).

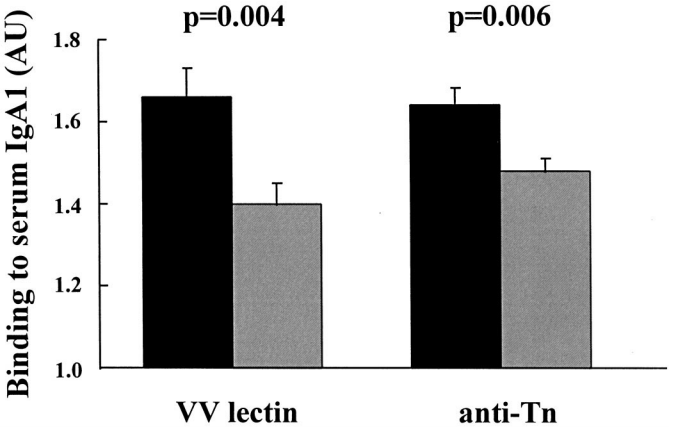

Figure 6. Vicia villosa (VV) lectin and anti-Tn binding to IgA1 in IgAN and controls. The binding of VV lectin and anti-Tn antibody to IgA1 from 20 IgAN and 20 controls was measured in an enzyme-linked immunosorbent assay-type system and expressed in arbitrary units. Results are shown as mean ± SEM. Black bars, IgAN; grey bars, control. IgA1 from patients with IgAN showed significantly higher binding of both VV lectin and anti-Tn than IgA1 from controls.

Correlation of FACE Analysis and VV Lectin/Anti-Tn Binding

In the 20 IgA1 samples from patients with IgAN that were used for these investigations, the percentage of single GalNAc glycans from FACE analysis showed good correlation with both VV lectin and anti-Tn binding of the same sample (%GalNAc *versus* VV $r = 0.78$, $P < 0.001$, %GalNAc *versus* Tn $r = 0.57$, $P = 0.009$) (Figure 7A). There was also a negative correlation between the percentage of Galβ1,3GalNAc on FACE gels and the VV lectin binding of IgA1 ($r = -0.64$, $P = 0.002$) (Figure 7B). No such correlations were found between FACE data and the VV or Tn binding of control IgA1.

Discussion

Altered *O*-glycosylation of IgA1 in IgAN is a physicochemical abnormality of the IgA1 molecule that may contribute to pathogenic mechanisms in a number of ways (24). However, analysis of IgA1 *O*-glycoforms is technically challenging, and the precise nature of the defect has proved difficult to elucidate. In this article, we present the first study of IgA1 *O*-glycans using the FACE technique. This method allowed us to simultaneously analyze all of the major *O*-glycan forms found in IgA1 for the first time.

Using FACE to analyze the *O*-glycans released intact from native serum IgA1, we found no difference between patients with IgAN and control subjects in the degree of sialylation of the galactosylated carbohydrate moieties, or of the relative frequencies of the sialylated *O*-glycans. However, in IgAN, we found a significant increase in the percentage of IgA1 *O*-glycans consisting of GalNAc alone, rather than the more usual galactosylated and sialylated chains. This provides powerful evidence that the well-described alterations of lectin binding to IgA1 in IgAN are due to increased occurrence of ungalactosylated GalNAc units rather than to *O*-sialylation changes. This may be due to an absolute increase in the number of *O*-glycans

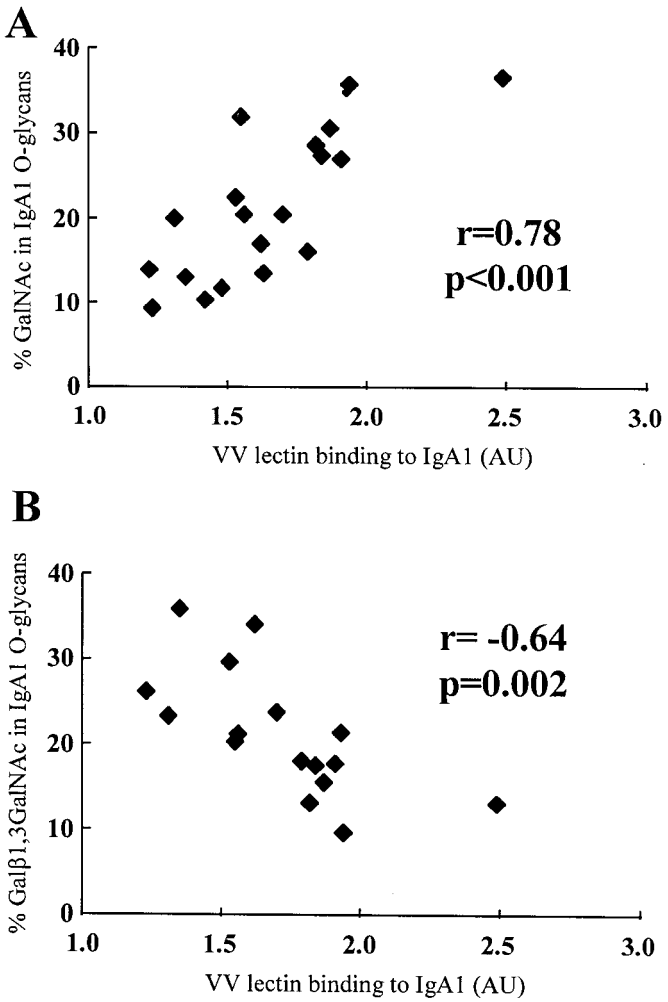

Figure 7. Correlation of FACE data and VV lectin binding to IgA1 in IgAN. FACE analysis of *O*-glycans released from native IgA1 by hydrazinolysis at 60°C in 20 patients with IgAN, and VV lectin binding of same IgA1 samples. (A) Positive correlation between %GalNAc from FACE analysis and GalNAc-specific VV lectin binding ($r = 0.78$, $P < 0.001$). (B) Negative correlation between %Galβ1,3GalNAc from FACE analysis and VV lectin binding ($r = -0.64$, $P = 0.002$). No such correlations between FACE analysis and lectin binding were seen in IgA1 from control subjects.

carried by IgA1, with the excess moieties consisting of single GalNAc units, or it may be due to reduced frequency of *O*-glycan galactosylation. The lack of a difference between IgAN and control subjects in the frequency of any of the galactosylated moieties B through D may tend to argue for the former possibility. However, the increased GalNAc percentage in IgAN may be due to a small decrease in each of the three galactosylated moieties, with these individual differences being too small to be apparent in the data analysis. In IgAN, we found good correlation between the frequency of glycan chains consisting of GalNAc alone and VV lectin binding of the same IgA1 sample, while the frequency of Galβ1,3GalNAc chains had a negative correlation with the binding of this lectin. VV lectin recognizes terminal GalNAc units (25,26), and this concurrence with our FACE findings lends strong support to its

reliability as a marker of IgA1 *O*-glycan agalactosylation in IgAN. Similar results were seen with the anti-Tn monoclonal antibody, which shares the same specificity as VV lectin (13). Therefore, this study confirms that abnormal IgA1 *O*-glycosylation in IgAN takes the form of increased occurrence of agalactosyl GalNAc units. Interestingly, there was no correlation between lectin or anti-Tn binding and FACE data in the control IgA1 samples. Presumably, exposure of terminal GalNAc units is sufficiently infrequent in control subjects for other factors to have more influence on lectin binding, and this observation suggests that the aberrant *O*-glycoforms seen in IgAN have the potential to significantly affect IgA1 interactions with other molecules.

IgAN is a heterogeneous disease, and few reported abnormalities are consistently seen in all patients. Altered IgA1 glycosylation is no exception in this respect; we found a wide range in the percentages of different *O*-glycan chains by FACE analysis and in the lectin binding values of both patients and control subjects, with considerable overlap between the groups. Defective *O*-glycosylation may be restricted to a subgroup of patients with IgAN, but it is not yet known whether this is associated with other IgA abnormalities or with disease progression or outcome.

We were not able to identify the two sialylated bands 1 and 3 with absolute certainty. Both bands were lost by digestion of IgA1 with α2,3-specific sialidase, indicating that they both contain sialic acid in this linkage. This observation, and examination of published data, led us to deduce that the upper band 1 is probably the disialylated form D, and the lower band 3 is the monosialylated glycan C. Absolute confirmation of this would require band excision and monosaccharide sequencing. However, there was no difference between patients and control subjects in the percentages of either of these bands, or in the ratio of sialylated:unsialylated chains.

In agreement with published data, we observed two bands (bands 5 and 6) that were not predicted from the reported *O*-glycan structures of IgA1. These migrated at the leading edge of the sample, and were probably small sugar units. Indeed, band 5 migrated in the same position as free galactose. Band 6 remains unidentified. These two bands occurred in glycan samples prepared from proteins carrying either *N*- or *O*-glycans, regardless of the method of sample preparation, and may indicate a degree of glycan degradation. However, sialic acid is the most labile and reactive of the *O*-glycan carbohydrate units, but resisted the preparation treatment sufficiently for sialylated chains to account for more than 50% of the *O*-glycans obtained from native IgA1 samples. The neutral core chains are perhaps even less likely to be degraded during this process. IgA1 samples from IgAN patients and control subjects were processed together in exactly the same way, and did not differ in their band 5 or 6 content. These considerations lead us to believe that our observations concerning *O*-glycan bands 1 to 4 are valid despite the uncertainty presented by these unidentified bands.

Whether the abnormally high occurrence of agalactosyl GalNAc in IgA1 in IgAN is an absolute increase, or whether it is due to reduced galactosylation of the same number of *O*-glycan chains is not yet clear. In either case, the reason for the apparent failure to galactosylate these moieties needs to be addressed and may result from a variety of mechanisms. The glycan chains may be truncated *in vivo* by circulating glycosidases. We have shown previously that this is unlikely, since in IgAN the *O*-glycosylation of serum C1inh is normal (2). We have also found that the *O*-glycosylation of IgA1 produced by peripheral blood B cells in culture parallels that of the serum IgA1 of the same individual (A. C. Allen, unpublished observations). Glycan chains are formed during the synthesis and posttranslational modification of glycoproteins by a series of highly specific intracellular enzymes, the glycosyltransferases. Aberrant IgA1 *O*-glycosylation may be due to an abnormal hinge region amino acid sequence presenting an altered template for the action of *O*-glycosyltransferases. Again, we have shown that this is unlikely, since the nucleotide sequence of the α1 hinge region gene is normal in IgAN (27). Alternatively, reduced galactosylation may be due to altered function of the relevant enzyme, β1,3 galactosyltransferase (β1,3GT), and indeed, we have described reduced activity of this enzyme in peripheral blood B cells in IgAN (28). Further investigation is now required to establish the underlying cause of this reduced enzyme activity and to identify the affected cells.

The functional effects of altered IgA1 *O*-galactosylation and their impact on the pathogenesis of IgAN are as yet speculative. The *O*-glycans of the IgA1 hinge region are recognized by the hepatic asialoglycoprotein receptor (29), which represents the major catabolic route of serum IgA1 (30). Lack of terminal galactose may therefore compromise clearance of abnormal IgA1 molecules, leading to their persistence in the circulation and eventual glomerular deposition. The hinge region lies in a pivotal position of the IgA1 molecule, between the antigen binding region of the Fab and the effector sites of the Fc portion. Closely located series of *O*-glycan such as those of the IgA1 hinge region tend to confer a rigid extended structure on the protein domain (31), while terminal sialylation bestows a negative charge on this otherwise neutral region of the molecule. Thus, truncation of the *O*-glycans has the potential to alter the three-dimensional shape and the charge of the IgA1 molecule, with possibly significant effects on stability (16), antigen binding, self-aggregation (32), and interactions with receptors, complement, and other proteins such as IgG (7) and extracellular matrix components (4).

Additional information is now required about the factors controlling the expression and activity of glycosyltransferases. Continuing investigations into the nature of the IgA1 *O*-glycosylation defect described here will help to elucidate whether the abnormality is peculiar to certain hinge region *O*-glycosylation sites, or restricted to certain populations of IgA1-producing cells. Additional studies into the functional effects of reduced IgA1 *O*-galactosylation in IgAN are also needed to establish the full relevance of this defect in the pathogenesis of this common glomerular disease.

Acknowledgments

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