

Galactosylation of N- and O-linked carbohydrate moieties of IgA1 and IgG in IgA nephropathy

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SUMMARY

The mechanism of IgA deposition in the kidneys in IgA nephropathy is unknown. Mesangial IgA is of the IgA1 subclass, and since no consistent antigenic target for the IgA1 has been described, we have investigated the glycosylation of the molecule, as a potential non-immunological abnormality which may contribute to its deposition. IgA1 is rich in carbohydrate, carrying N-linked moieties in common with IgG, but also O-linked sugars, which are rare in serum proteins, and not expressed by IgG or IgA2. Lectin binding assays were designed to examine the expression of terminal galactose on the N-linked carbohydrate chains of purified serum IgG and IgA1, and the O-linked sugars of IgA1 and C1 inhibitor (one of the very few other serum proteins with O-linked glycosylation). No evidence was found for abnormalities of N-linked glycosylation of either isotype in IgA nephropathy compared with matched controls. However, in IgA nephropathy, reduced terminal galactosylation of the hinge region O-linked moieties was demonstrated; this was not seen in C1 inhibitor, which showed normal or increased galactosylation of the O-linked sugars. This abnormality of IgA1 has considerable implications for the pathogenesis of IgA nephropathy, since the O-linked sugars lie in an important functional location within the IgA1 molecule, close to the ligand of Fc receptors. Changes in the carbohydrates in this site may therefore affect interactions with receptors and extracellular proteins, leading to anomalous handling of the IgA1 protein in this condition, including failure of normal clearance mechanisms, and mesangial deposition.

Keywords N-acetyl galactosamine T antigen lectins IgA nephropathy

INTRODUCTION

Mesangial IgA in IgA nephropathy (IgAN) is predominantly IgA1. Serum IgA1 is raised, but this does not explain why it deposits in the mesangium, since IgAN is not found in other conditions with raised circulating IgA1, such as HIV and IgA myeloma. No antigenic target for this IgA1 has been identified. Some non-immunological mechanism may be involved, due to a physicochemical abnormality of the IgA1 protein. One possible candidate is altered IgA1 glycosylation.

Glycoproteins undergo glycosylation as a mandatory post-translational modification. Mammalian protein glycosylation is found in two different linkages: N-linked to asparagine residues, and O-linked to serine or threonine residues. The carbohydrates form an integral part of the glycoprotein, helping to maintain the biologically active conformation, decreasing immunogenicity and protecting from proteolysis. They also act as ligands for cell receptors, enzymes, adhesion molecules and matrix components [1].

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In rheumatoid arthritis (RA) altered N-linked glycosylation of circulating IgG has been identified and correlated with disease activity. The N-linked moieties are branched chain sugars with distal N-acetyl glucosamine (GlcNAc), which may carry terminal D-galactose (D-Gal). In RA there is an increase in agalactosylated IgG, and reduced activity of the β 1,4 galactosyltransferase (β 1,4GT) necessary for terminal galactosylation of GlcNAc [2].

IgA1 is a heavily glycosylated molecule, carrying a number of N-linked glycosylation sites, of the same type as those found in IgG and many other serum proteins, and, unusually for a secreted protein, a series of O-linked moieties linked to serine residues in the hinge region. The hinge region is deleted in IgA2, which therefore lacks this type of glycosylation. One of the very few other O-galactosylated proteins found in appreciable concentrations in serum is C1 inhibitor (C1inh).

The O-linked carbohydrates which characterize the hinge region of IgA1 are simple compared with N-linked structures, consisting of O-linked N-acetyl galactosamine (GalNAc) with or without terminal D-Gal in the β 1,3 configuration (Gal β 1,3GalNAc, also referred to as the T antigen). Galacto-

sylated moieties can also carry sialic acid, a highly negatively charged entity which is much more labile than the core structure [3]. The lectin jacalin, known for its affinity for IgA1, binds the complete, galactosylated Gal β 1,3GalNAc moiety, and has been shown to have reduced affinity for serum IgA1 from patients with IgAN, suggesting some abnormality of the O-linked sugars of IgA1 in IgAN [4–6].

Lectins are proteins derived from plants or animals which show strong and specific affinity for certain carbohydrate ligands. This study used lectins specific for the various carbohydrate moieties of IgA1, IgG and C1inh, to look for abnormalities in serum immunoglobulins in IgAN.

SUBJECTS AND METHODS

Subjects

Serum was obtained with informed consent from patients with biopsy-proven IgAN attending the Nephrology Clinic at Leicester General Hospital, and controls recruited from hospital staff. For studies comparing IgA1 and IgG glycosylation, 20 patients (14 male; mean age 40 years, range 20–63 years) and 20 controls (14 male; mean age 39 years, range 21–62 years) were used. For studies comparing IgA1 and C1inh glycosylation, 12 patients (10 male; mean age 46 years, range 28–62 years) and 12 controls (nine male; mean age 33 years, range 25–42 years) were recruited. No patient had macroscopic haematuria or evidence of renal insufficiency at the time of study; all had microscopic haematuria \pm proteinuria. This study was approved by the Ethical Committee of Leicestershire Health Authority.

Purification of serum IgA1, IgG and C1inh

Immunoglobulin fractions were prepared by ammonium sulphate precipitation. Serum (5 ml) was diluted with an equal volume of PBS and 2.8 g ammonium sulphate gradually dissolved in it. After stirring to equilibrate, the samples were spun down hard, and the precipitate redissolved in the appropriate buffer for the following purification process. IgG was purified by combined gel filtration and ion exchange chromatography (adapted from [7]) with 5 g Sephadex G25 (Sigma, Poole, UK) in phosphate buffer pH 7.2 layered on top of 6 g DE52 ion exchanger (Whatman, Maidstone, UK). Ammonium sulphate precipitate samples (1 ml) were applied, washed through with phosphate buffer, collecting the second 10-ml fraction, containing IgG. This method harvested all four IgG subclasses in similar proportions to those of the original serum.

For IgA1 purification, ammonium sulphate precipitates in 0.175 M Tris-HCl buffer pH 7.5 were applied to washed jacalin agarose (Vector, Peterborough, UK) in centrifuge tubes and incubated at room temperature. After washing the agarose three times in Tris-HCl, IgA1 was eluted with 0.8 M galactose, and the buffer exchanged to PBS on Sephadex G25 columns to remove free galactose.

For purification of IgA1 and C1inh from the same serum samples, sequential precipitation with 20% and 40% polyethylene glycol 6000 (PEG) was performed. The precipitates were redissolved in Tris-HCl buffer and applied to jacalin agarose as above. IgA1 was obtained from the less soluble fraction (20% PEG), and C1inh from the more soluble fraction (40% PEG).

The purity of the samples was checked by SDS-PAGE and Western blotting, and the IgA, IgG and C1inh concentrations

were measured using sandwich ELISA. The samples were stored in aliquots at -20°C until required.

Enzyme-linked lectin binding assays

Terminal agalactosylation of the N-linked moieties of IgA1 and IgG was assessed by the relative binding of the lectins from *Triticum vulgare* (TV) (Sigma), which binds terminal GlcNAc [8], to *Erythrina cristagalli* (EC) (Sigma), which binds terminal D-Gal of N-linked moieties [8] (method described in [9]). Immunoplates were saturated with IgG or IgA1 samples in duplicate wells overnight at 4°C , bound immunoglobulin denatured with 0.5% SDS to fully expose the glycosylated sites, and excess protein binding capacity blocked with oxidized glutathione. Biotinylated lectins were applied (TV 1 $\mu\text{g}/\text{ml}$; EC 2 $\mu\text{g}/\text{ml}$), and binding visualized with horseradish peroxidase (HRP)-conjugated avidin D (Vector) (2.5 $\mu\text{g}/\text{ml}$) and OPD/ H_2O_2 chromogenic substrate. Optical density at 492 nm was read on a Titertek Multiscan plate reader. Results were expressed as ratios of TV:EC absorbance (A) at 492 nm.

O-linked carbohydrates of the hinge region of IgA1 were measured in a similar assay. Biotinylated lectins from *Helix aspersa* (HA) (Sigma) (2 $\mu\text{g}/\text{ml}$) and *Vicia villosa* (VV) (Vector) (1 $\mu\text{g}/\text{ml}$) were used to bind ungalactosylated GalNAc [8,10] and lectins from *Amaranthus caudatus* (AC) (0.5 $\mu\text{g}/\text{ml}$) and *Arachis hypogaea* (peanut agglutinin (PNA)) (Vector) (2.5 $\mu\text{g}/\text{ml}$) to detect the complete, galactosylated Gal β 1,3GalNAc moiety [8]. Denaturation of IgA1 with 0.5% SDS, 5 M urea at pH 2.5, or boiling, did not alter lectin binding, so this stage was abandoned. To compare the O-linked sugars of IgA1 and C1inh, HA and VV lectins were used in similar assays. Results were expressed as A at 492 nm.

All the samples were always assayed for each lectin in a single run, so interassay variability did not apply. The assays were repeated on several different occasions, and the same patterns obtained each time. The results were analysed using unpaired *t*-tests to compare IgAN and control data for the binding of each lectin.

RESULTS

Purification of IgA1, IgG and C1inh (Fig. 1)

An SDS-PAGE of purified IgA1 samples is shown in Fig. 1. IgG and C1inh purification gave similar degrees of purity. Typical yields were 500 μg IgA1, 5 mg IgG, and 200 μg C1inh per ml of serum.

N-linked galactosylation of IgG and IgA1

There was no difference in the TV:EC binding ratio of IgG or IgA1 from patients with IgAN compared with controls (data not shown). There was therefore no evidence of altered terminal galactosylation of the N-linked sugars in IgAN.

O-linked galactosylation of IgA1 (Fig. 2)

Serum IgA1 from patients with IgAN showed significantly higher binding than controls to HA and VV lectins, specific for O-linked GalNAc. No difference was seen in binding to AC and PNA lectins, specific for the fully galactosylated Gal β 1,3GalNAc. This latter finding may be an anomaly due to the use of jacalin isolation, since jacalin binds the Gal β 1,3GalNAc moiety and would therefore tend to select for molecules rich in this moiety.

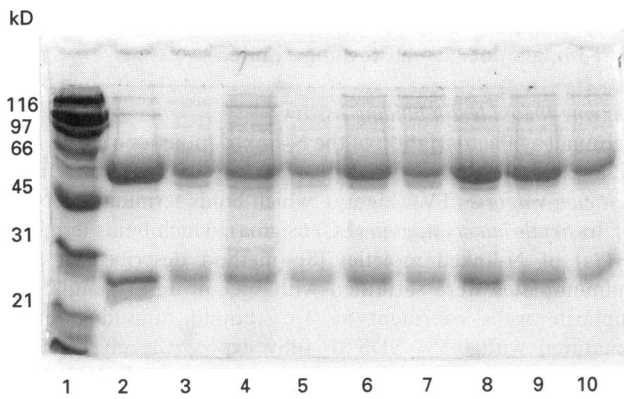


Fig. 1. Typical SDS-PAGE of reduced IgA1 samples, stained with coomassie blue. Lane 1, molecular weight marker; lane 2, commercially purified IgA1 myeloma protein (The Binding Site, Birmingham, UK); lanes 3–10, IgA1 samples purified from IgA nephropathy (IgAN) and control sera. IgA1 has dissociated into α 1 heavy chains at 56 kD and light chains at 25 kD.

O-linked galactosylation of C1inh (Fig. 3)

In a second set of sera from 12 patients and 12 controls, IgA1 binding of HA and VV lectins was again significantly higher in IgAN. C1inh from these sera showed no difference in C1inh binding of HA lectin between IgAN and control, but significantly lower binding of C1inh from IgAN to VV lectin than control.

DISCUSSION

The mechanism of mesangial IgA1 deposition in IgAN remains unknown, but could be explained by altered IgA1 glycosylation. We found no evidence in IgAN for alterations in N-linked glycosylation of either IgA1 or IgG, as has been described in RA, but we did find decreased galactosylation of O-linked moieties of IgA1.

O-linked sugars are highly expressed in some membrane

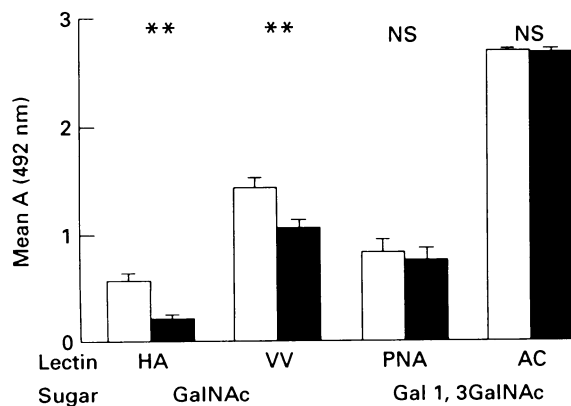


Fig. 2. Binding of lectins specific for O-linked sugars to serum IgA1 from patients with IgA nephropathy (IgAN; ■) and controls (□); mean + s.e.m. *Helix aspersa* (HA) and *Vicia villosa* (VV) lectins, specific for ungalactosylated N-acetyl galactosamine (GalNAc) showed significantly higher binding to IgA1 from IgAN than controls. ** $P < 0.001$. PNA, Peanut agglutinin; AC, *Arachis hypogaea*.

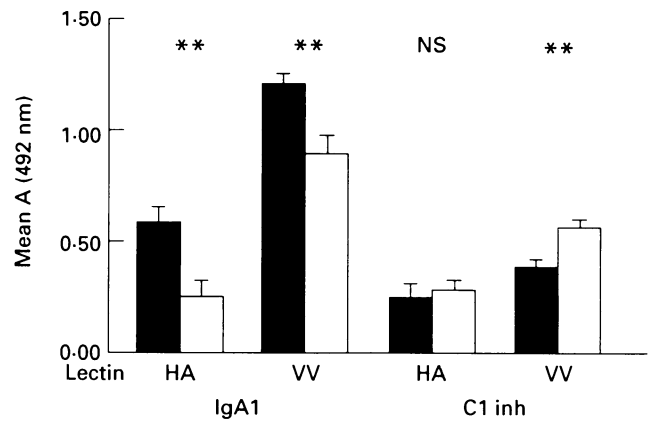


Fig. 3. Binding of lectins specific for O-linked sugars to serum IgA1 and C1 inhibitor (C1inh) from patients with IgA nephropathy (IgAN; ■) and controls (□); mean + s.e.m. *Helix aspersa* (HA) and *Vicia villosa* (VV) lectins (specific for ungalactosylated N-acetyl galactosamine (GalNAc)) showed significantly higher binding to IgA1 from IgAN than controls. HA lectin showed no difference in binding to C1inh between IgAN and control, while VV lectin showed significantly lower binding to C1inh from IgAN. ** $P < 0.005$.

proteins, but rare in serum proteins. IgA1 is the only major serum immunoglobulin possessing them—the hinge region is deleted in IgA2, which therefore lacks them. The decreased binding of IgA1 in IgAN to jacalin [4–6] is almost certainly explained by altered hinge region glycosylation, as may be the restricted charge of eluted mesangial IgA1 [11] and the altered pI of circulating IgA1 [12,13]. Altered glycosylation of the flexible hinge region may affect the 3-D structure of the IgA1. The hinge is also close to the ligand for cellular $Fc\alpha$ receptors, at the top of the CH2 domain, and this receptor–ligand interaction can be influenced *in vitro* by modifications of hinge region glycosylation [14,15]. This may be relevant to mesangial deposition and clearance of IgA1, since $Fc\alpha R$ have recently been identified on human mesangial cells [15,16]. Sugar–lectin interactions may also be important in the binding of IgA1 to extracellular matrix proteins [17]. Clearance of IgA1 from the circulation is mediated by O-linked sugars, since terminal O-linked D-galactose is the ligand of the hepatic asialoglycoprotein receptor (ASGPR). The persistence of IgA immune complexes in the circulation in IgAN, despite normal clearance of other ligands of the ASGPR [18], suggests an abnormality of IgA1 which inhibits its interaction with the ASGPR, consistent with the terminal agalactosylation of the O-linked sugars described here.

We found no difference in IgA1 binding to two lectins with affinity for the galactosylated moiety, contrasting with the results of others [4–6], who found a decrease in IgA1 binding to jacalin, which also recognizes the galactosylated form. However, in our study jacalin was necessarily used to isolate IgA1 from serum, thus selecting only the molecules bearing galactosylated forms. Despite this selection for 'normally' galactosylated hinge region sugars, we were able to show a very marked reduction in the amount of terminal galactosylation in IgAN, and we would predict that the molecules with least galactosylation were lost in the purification process. Any IgA1 with unusually strong binding to jacalin may also have

been lost if it resisted the elution conditions used. It is possible that our approach has underestimated the abnormality of O-linked glycosylation of IgA1, particularly in view of the reported differences in jacalin binding of IgA in IgAN [4]. At present jacalin is necessary for IgA1 isolation from whole serum; affinity chromatography using capture by anti-IgA1 requires harsh treatment for subsequent dissociation of IgA1 from the antibody, which renders it unsuitable for glycosylation assays.

Normal serum IgA1 carries terminal sialic acid on both N- and O-linked sugars. Sialylation was not addressed in this study; however, the denaturation step employed in some of the assays would also be likely to remove the labile sialic acid from the sugars. The differences between IgAN and control in binding of lectins to IgA1 were the same after this treatment, suggesting that the abnormality lies within the core sugar.

Using fractionation of oligosaccharides released by hydra-zinolysis, and matrix-assisted laser desorption ionization-time of flight mass spectrometry, Hiki *et al.* [19] have shown altered oligosaccharide proportions consistent with our data. However, these techniques analyse oligosaccharides chemically liberated from the carbohydrate chains, and cannot show the way the abnormality is expressed in its native, biological form. The lectin binding approach used in our study is not only simple, rapid and inexpensive in comparison with other methods, but also allows analysis of the relative occurrence of different moieties built up from these individual oligosaccharides *in situ*.

The underlying mechanism of this reduced terminal galactosylation is as yet unknown, but it may be due to reduced activity of the intracellular enzyme β 1,3 galactosyl-transferase (β 1,3GT) [6]. There is an interesting parallel in the rare haematological disorder, Tn polyagglutinability syndrome, in which red cell membrane proteins fail to express the normal carbohydrate Thomsen-Friedenrich ('T') antigen, which is known to be O-linked Gal β 1,3GalNAc, leading to the exposure of GalNAc, which acts as a neoantigen, 'Tn'. Since normal sera contain an autoantibody to Tn, this results in red cell agglutination. This failure of terminal galactosylation is identical to that we have described in IgAN. In the Tn polyagglutinability syndrome, expression of the Tn antigen results from reduced β 1,3GT activity in affected cells [20–24]. Therefore, this galactosylation defect has been described in membrane proteins; but not previously in a serum protein. Our finding of normal or decreased binding of Clinh to lectins specific for ungalactosylated GalNAc in IgAN suggests that this protein has normal or increased terminal galactosylation of the O-linked sugars. The failure of galactosylation of IgA1 would appear to be a defect restricted to certain cell types, rather than being a widespread phenomenon.

Altered O-linked glycosylation of the hinge region of IgA1 is an important new factor in the pathogenesis of IgAN. The underlying mechanism and functional consequences of this abnormality require further study.

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