O-Glycosylation of Serum IgD in IgA Nephropathy

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In IgA nephropathy (IgAN), serum IgA1 with abnormal O-glycosylation preferentially deposits in the glomerular mesangium. The control of O-glycosylation is poorly understood. Among Ig isotypes, only IgD, produced early in B cell development, and IgA1, produced by mature B cells, are O-glycosylated. For investigation of the stage of B cell maturation at which the defect seen in IgAN arises, the O-glycosylation of serum IgA1 and IgD was studied in IgAN and controls. Serum was obtained from 20 patients with IgAN and 20 control subjects. The O-glycosylation profiles of native and desialylated IgA1 and IgD were measured in an ELISA-type system using the lectins Helix aspersa and peanut agglutinin, which bind to alternative forms of O-glycan moieties. The lectin-binding patterns of the two immunoglobulins differed in all participants, with that of IgD suggesting that it is more heavily galactosylated than IgA1. Defective O-glycosylation of IgA1, probably taking the form of reduced galactosylation, was confirmed in IgAN in this study. This undergalactosylation was not shared by IgD; in contrast, IgD carried more galactosylated O-glycans in IgAN than controls. The contrasting lectin-binding patterns of IgA1 and IgD shows that O-glycosylation is differentially controlled during B cell maturation. Compared with controls, O-glycosylation in IgAN is incomplete in IgA1 but more complete in IgD. These observations show that abnormal IgA1 O-glycosylation in IgAN is not due to an inherent defect in glycosylation mechanisms but arises only at a later stage in B cell development and may be secondary to aberrant immunoregulation.

IgD, like IgA1, has a hinge region, and although the amino acid sequences of the two isotypes differ (20,21), they both carry approximately five O-glycan moieties per heavy chain. Hitherto, the O-glycosylation of IgD in IgAN has not been investigated, but this would be informative, as demonstration of an abnormality in both IgA1 and IgD O-glycosylation would suggest that the defect arises early in B cell development and may affect all B cells, whereas its restriction to IgA1 alone would indicate a later and possibly population-specific origin.

We have studied the O-glycosylation profiles of IgA1 and IgD from patients with IgAN and matched control subjects using our previously published lectin-binding assays. In both patients with IgAN and control subjects, we found distinct differences in the lectin-binding patterns of IgA1 and IgD, indicating that IgD is more heavily galactosylated but less sialylated than IgA1. As we and others have reported before (22), in patients with IgAN, IgA1 O-glycosylation was abnormal, with lower terminal galactosylation than in control subjects. However, the lectin-binding patterns of IgD suggested either a higher degree of terminal galactosylation or a higher density of O-glycan chains per molecule in patients with IgAN. These results show that the abnormality of IgA1 O-glycosylation in IgAN is not shared by IgD and is not due to defective expression or function of glycosylating enzymes that affect the entire B cell lineage. It seems that the pattern of Ig O-glycosylation is differentially controlled at different stages of B cell development and suggests that the alterations that are seen in IgAN may be secondary to aberrant immunologic control mechanisms.

Materials and Methods

We studied 20 patients with biopsy-proven IgAN (17 male; median age 40 [range 20 to 69 yr]) and 20 healthy control subjects with no history of kidney disease (17 male; median age 36 [range 20 to 55 yr]). All participants gave informed consent for inclusion in the study. At the time of the study, no participant had an intercurrent illness, and none of the patients with IgAN had macroscopic hematuria. The median time from renal biopsy to entry into the study for patients with IgAN was 4 yr (range 0.5 to 6). All patients with IgAN had microscopic hematuria and/or proteinuria, but proteinuria was 2.5 g/24 h in all cases. The median serum creatinine in the patients with IgAN was 125 μmol/L (range 64 to 146). None of the patients or control subjects was receiving immunosuppressive treatments at the time of the study or had received treatment in the 24 mo before study entry. All participants gave their written informed consent, and the study was approved by the Leicester Research Ethics Committee.

Materials

Unless otherwise stated, all reagents and materials were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Polyclonal anti-IgA, anti-IgD, and horseradish peroxidase (HRP)-conjugated anti-mouse antibodies; monoclonal anti-IgD; and 1,2-phenylenediamine dihydrochloride (OPD) substrate tablets were obtained from Dako Ltd (Ely, UK).

Samples

Venous blood was obtained, and serum was separated and stored in aliquots at −80°C until required.

Measurement of Serum IgA1 and IgD Concentrations by ELISA

Rabbit anti-human antibodies to IgA and IgD were diluted to 10 μg/ml in 0.05 M carbonate/bicarbonate buffer (pH 9.6), as recommended by the supplier and found to be optimal in preliminary titration experiments in our laboratory. A total of 100 μl/well was applied as capture antibody to 96-well immunoplates (Nunc Immunoplate, Life Technologies, Renfrew, UK) and incubated at 4°C for at least 24 h
before use. The plates were washed four times with washing buffer (PBS/0.3 M NaCl/0.1% Tween 20), and excess protein-binding sites were blocked with 100 μl/well 2% BSA in PBS for 1 h at room temperature. After further washing, 50-μl aliquots of standard and test serum samples, diluted in PBS, were applied to duplicate wells. Standard curves were set up on each plate, using serial dilutions of a commercial human Ig calibrator preparation (The Binding Site, Birmingham, UK) ranging from 1 μg/ml to 1 ng/ml IgA1 or IgD. Serum samples were used at preoptimized dilutions for each assay. The plates were sealed and incubated at 4°C overnight.

The plates were washed again, and bound IgA1 and IgD were detected with 50 μl/well mAb to IgA1 (1:1000) and IgD (1:250), respectively. After incubation for 90 min at room temperature and washing again, 50 μl/well HRP-conjugated anti-mouse Ig antibody at 1:1000 in PBS was applied to the plates and incubated for an additional 90 min at room temperature. Finally, the plates were washed again and developed with OPD substrate at 50 μl/well. The reaction stopped when the color reached an appropriate intensity with 75 μl/well 1 M H₂SO₄, and the results were read using an automated plate reader with a 492-nm filter (Titertek Multiscan, ICN Flow, High Wycombe, UK). Means of duplicate wells were calculated, and a standard curve was constructed for each plate. Sample values were read from the standard curves, excluding samples that did not fall on the linear part of the curves; these were repeated at a more suitable dilution.

**Lectin-Binding Assays**

IgA1 and IgD O-glycosylation patterns in different serum samples were compared using our previously published ELISA-type lectin-binding assays (22,23). The lectins that were used in this study were *Helix aspersa* (HA) and peanut agglutinin (PNA). HA binds to terminal GalNAc, and high binding of this lectin indicates a low degree of galactosylation and sialylation of the O-glycan moieties. PNA binds to the core 1 disaccharide Gal-GalNAc and is extremely inhibited by the presence of sialic acid.

Immunoplates were coated with anti-IgA or anti-IgD and blocked with BSA as described for ELISA above. Test serum samples were diluted in PBS, and 50 μl/well was applied to duplicate wells on a series of replicate immunoplates. After overnight incubation and further washing, 50 μl/well biotinylated HA and PNA lectins were applied to replicate plates at 1:250 and 1:500 in PBS, respectively, incubated for 90 min at room temperature, and washed again, and lectin-binding was detected with 50 μl/well HRP-conjugated avidin for 90 min at room temperature. The results were developed with OPD substrate and stopped with 1 M H₂SO₄ as for ELISA above. We previously showed that for IgA1, lectin binding is proportional to IgA1 over a concentration range of 0.1 to 100 μg/ml (23). In preliminary experiments for this study, this was shown to be true also for IgD lectin binding. Therefore, the test samples that were used in these assays were diluted to achieve IgA1 and IgD concentrations of approximately 1 μg/ml, and replicate plates were developed with HA and PNA lectins and also with anti-IgA1 or anti-IgD as appropriate, followed by anti-mouse-HRP as for ELISA. The results were expressed as mean absorbance at 492 nm of duplicate wells, with each lectin-binding result adjusted for IgA1 or IgD binding absorbance to correct for minor variations in the Ig concentrations of the different samples.

**Desialylation of IgA1 and IgD**

The presence of terminal sialic acid moieties on the O-glycans of IgA1 and IgD can mask lectin binding to the inner chain and confuse interpretation of the results of these assays. Therefore, we compared lectin binding of native and desialylated IgA1 and IgD. Replicate immuno-plates were coated with primary antibodies and blocked, and diluted serum samples were applied as described above. After allowing capture of IgA1 or IgD for 24 h at 4°C, one set of plates was washed and desialylated with 50 μl/well *Clostridium perfringens* neuraminidase (New England Biolabs, Hitchin, UK) at 100 U/ml in 50 mM sodium citrate buffer (pH 6.0) for 18 h at 37°C. A parallel set of plates were left untreated at 4°C as native Ig. After this step, the lectin-binding assays were continued exactly as described above.

**Statistical Analyses**

Serum IgA1 and IgD concentrations and lectin-binding results for the IgAN and control groups were expressed as mean ± SEM. Unpaired t test were used to compare the two subject groups, and paired t test was used to compare the lectin binding of native and desialylated IgA1 and IgD within the subject groups.

**Results**

**Serum IgA1 and IgD**

There was no statistically significant difference between the IgAN and control groups in the serum concentrations of IgA1 or IgD (Figure 2).

**Comparison of IgA1 and IgD O-Glycosylation Profiles in Control Subjects**

In both control subjects and patients with IgAN, there were marked differences in the lectin-binding patterns of IgA1 and IgD, demonstrating that these two O-glycosylated Ig have characteristic O-glycan chain types. Figure 3 compares the lectin binding of the two Ig from control subjects: The same pattern was seen in IgAN. As we previously reported and confirm here, native IgA1 shows significant HA binding, but its PNA binding is very low or even at background levels. PNA binding to its ligand Gal-GalNAc is exquisitely sensitive to the presence of sialic acid, and its failure to bind to native IgA1 demonstrates appreciable sialylation of the O-glycans. This is illustrated by the marked increase in PNA binding to desialylated IgA1 (10-fold increase over native IgA1). HA binds to ungalactosylated

![Figure 2](Image)
GalNAc units. It also is inhibited by sialic acid to some degree, as its binding shows a 3.6-fold increase after IgA1 desialylation, although this is less inhibition than is seen with PNA. Taken together, these data show that IgA1 carries a mixture of single GalNAc units and Gal-GalNAc chains and that many of these moieties are sialylated.

Relative to IgA1, both native and desialylated IgD had lower HA binding. HA binding to IgD shows only a twofold increase after desialylation, suggesting that IgD has fewer O-glycans that consist of GalNAc alone than does IgA1. However, the binding of PNA to native IgD was higher than that of native IgA1. Because this lectin is exquisitely sensitive to the presence of sialic acid, this observation indicates a higher occurrence of asialyl O-glycans in IgD than in IgA1. Desialylation of IgD results in an additional 10-fold increase in PNA binding and further exaggerates its higher binding to IgD than to IgA1, demonstrating a higher frequency of Gal-GalNAc in IgD, in agreement with the HA results.

Lectin Binding to IgA1 in IgAN
In agreement with our previous reports, HA binding to both native and desialylated IgA1 was higher in patients with IgAN than in control subjects. PNA binding to native IgA1 was also higher than in control subjects, but in this case, the difference between the subject groups was lost on desialylation. These observations suggest that the increased HA binding in IgAN is due to increased frequency of IgA1 O-glycan moieties that consist of GalNAc alone, whereas the increased PNA binding to native IgA1 can be attributed to lower sialylation in IgAN, as this difference is lost on desialylation. The lower sialylation may be secondary to reduced galactosylation, as the galactosylated form has the potential to carry two sialic acid units per chain, and it is likely that most sialic acid units are carried on the external Gal unit rather than the inner GalNAc (Figure 4).

Lectin Binding to IgD in IgAN
There was no difference between patients with IgAN and control subjects in the binding of HA lectin to native or desialylated IgD, showing that IgD does not share the reduction in terminal O-galactosylation found in IgA1 in IgAN. However, PNA binding to IgD was higher in IgAN. This increased binding cannot be attributed to altered sialylation in IgD, as it persisted after desialylation, but indicates either that the O-glycans of IgD are more heavily galactosylated in IgAN or that each IgD molecule carries more O-glycan residues (Figure 5).

Discussion
In this study we used lectin-binding assays to compare the O-glycosylation patterns of serum IgA1 and IgD from patients with IgAN and control subjects. We confirmed the previously reported abnormality of O-glycosylation of serum IgA1 in IgAN detected by increased binding of the GalNAc-specific lectin HA, which suggests undergalactosylation of the core O-glycan chains. We found that in both patients with IgAN and control subjects, serum IgD has a distinct and different pattern of O-glycosylation to IgA1, IgD being more heavily galactosylated but less sialylated. There was no evidence for IgD undergalactosylation in IgAN; conversely, the lectin-binding patterns suggested that compared with control subjects, IgD in patients with IgAN either is more galactosylated or carries more O-glycan moieties per molecule.

In this study, we used lectin binding to assess the composition of the O-glycan chains of IgA1 and IgD. This is a somewhat imprecise method of glycosylation analysis, as the specificity of the lectins is not absolute and their binding may be affected by physicochemical factors other than the target sugar moiety. Lectin binding cannot provide detail about the precise structures of the O-glycans but can be used to identify different overall patterns of O-glycosylation. These assays have been used widely for IgA1 O-glycosylation analysis as they are straightforward, require only small quantities of serum, and
can be applied to many samples in a single run (22). Such assays consistently demonstrate the O-glycosylation abnormality of serum IgA1 in IgAN, and although they cannot define the exact structural abnormality, they correlate well with other, more precise analytical methods, such as carbohydrate electrophoresis (6) and mass spectrometry (24). Therefore, they can be used confidently to compare the O-glycosylation profiles of series of samples. The more precise methods present technical and interpretative difficulties that have yet to be completely overcome, and they require complex purification and preparation steps that introduce their own potential sources of error, including the possible loss of some relevant minority glycoforms. This study presents the first use of O-glycan–specific lectins for IgD glycosylation analysis. Further structural analysis now is required but must await the development of methods for reliable separation of serum IgD from IgA1 before adaptation of mass spectroscopy or carbohydrate electrophoresis techniques. The low level of IgD in serum will render this particularly challenging.

IgD is primarily a membrane-bound Ig that first is expressed by mature but naïve B cells when they leave the bone marrow and that frequently is co-expressed with surface IgM (17). When antigen is encountered and activation occurs, most B cells cease to express IgD and mature into IgM plasma cells or class-switch to IgG or IgA production. Once this has occurred, IgD expression is irrevocably lost: The memory B cell population contains no IgD cells (19). However, a few of the early

Figure 4. Lectin binding to the O-glycans of IgA1 in patients with IgAN and control subjects. Binding of the lectins HA (top) and PNA (bottom) to native and desialylated IgA1 from patients with IgAN (○) and control subjects (□). In patients with IgAN, both native and desialylated IgA1 show higher HA binding than in control subjects, indicating a higher frequency of agalactosyl GalNAc units. PNA binding to native IgA1 is also higher in patients with IgAN, although it is extremely low in both subject groups. After IgA1 desialylation, this difference between the subject groups is lost, indicating that it was due to lower sialylation rather than higher galactosylation in patients with IgAN.

Figure 5. Lectin binding to the O-glycans of IgD in patients with IgAN and control subjects. Binding of the lectins HA (top) and PNA (bottom) to native and desialylated IgD from patients with IgAN (○) and control subjects (□). There was no difference between patients and control subjects in HA binding to native or desialylated IgD, showing that IgD does not share the characteristic O-glycosylation defect displayed by IgA1 in patients with IgAN. PNA binding to native IgD was higher in patients with IgAN than in control subjects. Because this difference persists after IgD desialylation, it cannot be attributed to altered sialylation and must be due to an increase in the occurrence of galactosylated O-glycan forms in IgD in IgAN.
activated B cells lose IgM instead and mature into plasma cells that secrete IgD antibodies as part of the primary immune response and are the source of serum IgD.

IgA production occurs only after antigen encounter and activation and represents a more advanced stage of B cell development associated with somatic hypermutation and class switching. Like IgG, IgA is produced in the later stages of primary immune responses and by memory B cells during secondary responses. The majority of IgA is produced in the mucosa and is transported directly across the epithelial barrier into external secretions without entering the blood (3). Mucosally secreted IgA consists of approximately equal quantities of IgA1 and IgA2 and is almost exclusively polymeric. By contrast, serum IgA is predominantly monomeric and of the IgA1 subclass and is produced by plasma cells that are located in the bone marrow and peripheral immune sites, most notably the tonsils.

IgA1 and IgD are unique among human Ig in their possession of a hinge region decorated with a series of approximately five O-glycans (2–4,14–16). The O-glycan chains probably help to maintain the extended structure of the molecules, holding the Fc portion spatially distant from the Fab, and also confer some protection of the hinge region, which is vulnerable to digestion by specific proteases that are produced by a number of pathogenic microbes (3,17).

The O-glycan chains of both IgA1 and IgD are of the type known as core 1 structures, based on the disaccharide Galβ1–3GalNAc (25). Synthesis of these moieties takes place in the Golgi apparatus and is effected by the sequential actions of highly specific glycosyltransferases, which have only recently begun to be recognized and understood. The initial event is the addition of GalNAc to threonine or serine in the protein backbone, mediated by a UDP-N-acetyl-α-d-galactosamine:polypeptide N-acetylgalactosaminyltransferase (pp-GalNAc-Ts). A family of these enzymes has been described, only one of which, named pp-GalNAc-T2, seems to have significant function in human IgA1 B cells (26); IgD B cells have not been studied in this regard. The galactosylation of the core 1 structure then is completed by a core 1 β1 to 3 galactosyltransferase, C1Gal-T1 (25). It is interesting that a C1Gal-T1 activity requires the co-expression of a chaperone protein Cosmc, the expression of which may be a limiting factor in the function of the galactosyltransferase (27). Finally, O-sialylation may occur, catalyzed by members of the sialyltransferase family (28).

Hitherto, most of the available evidence points at undergalactosylation of the IgA1 hinge region O-glycans. It has been proposed that this defect arises from compromised expression or function of C1Gal-T, although, until recently, studies have been hampered by difficulties in isolating this enzyme. A similar undergalactosylation of the core 1 O-glycans of cell membrane proteins, leading to the expression of the so-called Tn antigen (GalNAc alone), is characteristic of some cancers and of the rare hematologic disorder Tn syndrome (29). This has been shown to be due to total lack of C1Gal-T activity in cells of the affected clones, whereas in the Jurkat cell, a Tn-expressing T cell line, the failure of C1GaLT activity is due to a mutation in the gene for the chaperone protein Cosmc rather than in the enzyme itself (27).

In IgAN, there is no evidence for undergalactosylation of O-glycosylated proteins unrelated to IgA1; for example, the lectin binding of C1 inhibitor, another serum protein that carries core 1 O-glycans, is normal in IgAN (5). The current study extends this observation considerably, showing that patients with IgAN are able to O-glycosylate IgD molecules adequately and suggesting that if defective enzyme activity is responsible, then it does not affect all B cells but is limited to a population that produces IgA1. Therefore, there is no global defect in expression of any of the O-glycosylating enzymes or the chaperone protein Cosmc in IgAN.

We found that the lectin-binding patterns of IgA1 and IgD were distinctly different from one another, IgD being more heavily galactosylated but less sialylated than IgA1 in all of the individuals studied. This finding was in agreement with a previous study (14) of IgD O-glycans, which showed that approximately 50% of the IgD O-glycans were asialyl Gal-GalNAc chains and the remainder were mono- or di-sialylated forms of the same core structure. Noagalactosyl GalNAc moieties were found. By contrast, studies of the IgA1 O-glycans always report a significant proportion of agalactosyl GalNAc units and find that the majority of completed core 1 structures are sialylated. This differential O-glycosylation of the two Ig isotypes show that Ig O-glycosylation is tightly regulated and varies during the development of an immune response, presumably as a result of controlled alterations in the expression or activity of glycosyltransferases. As IgD, produced early in the development of an immune response, is more completely O-glycosylated, the differential activity of the glycosyltransferases cannot be due simply to immaturity of the cells. Conversely, galactosylation seems to be downregulated in the more mature IgA1-secreting cells. It is possible that the rate of IgA1 synthesis exceeds the galactosylating capacity of the plasma cells, and it is intriguing to speculate that the availability of the chaperone protein Cosmc may be a factor in this. In contrast, IgA1 is more sialylated than IgD, indicating upregulation of sialyltransferases with maturation.

The O-glycosylation of serum IgA1 is highly variable. Mass spectrometric analysis of hinge region glycopeptides has shown that in each individual serum sample, between 10 and 20 different glycosylated peptides are represented in significant proportions (30). This array of O-glycoforms may reflect diversity of immune activity, with each IgA1 plasma cell producing antibodies that are O-glycosylated according to the nature and the maturity of the immune response involved.

In patients with IgAN, the contrast in the completeness of O-galactosylation between IgA1 and IgD is even more marked than in control subjects, with IgD being more galactosylated and IgA1 less so. Because it is clear that these patients do not lack the cellular machinery for full galactosylation of Ig O-glycans, the aberrant galactosylation points to some alteration in the state or control of the IgAN immune system. As alluded to above, a higher rate of IgA1 synthesis may outstrip the availability of C1Gal-T or Cosmc. Alternatively, this low galactosylation may be entirely appropriate and normal for IgA1 molecules in some immunologic situations, and the increased
frequency of such glycoforms in serum may reflect an unusual predominance of a particular type or stage of immune activity. Aberrations in immune function are recognized in IgAN. Circulating T cells display increased activation and cytokine production (31–36), and there is persistent systemic overproduction of plgA1 against a variety of systemic and mucosal antigens (37–39), but this is associated with a failure of affinity maturation of serum IgA antibodies (40). In the mucosa, subclinical intestinal inflammation is found in IgAN (41,42), but mucosal plgA1 plasma cell numbers (42,43) and mucosally secreted IgA antibody responses (44) are reduced. Taking this evidence together, a picture emerges of a subtle immune overactivity in which the IgA arm of the immune response fails to function properly. The underlying reason for this remains a mystery, but the dysregulation of IgA immune responses in IgAN deserves further study, for it is likely that altered O-glycosylation is one of the consequences of these disturbances.

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References
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Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/