O-Linked Glycosylation Determines the Nephritogenic Potential of IgA Rheumatoid Factor

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ABSTRACT

Deficient glycosylation of O-linked glycans in the IgA1 hinge region is associated with IgA nephropathy in humans, but the pathogenic contribution of the underlying structural aberrations remains incompletely understood. We previously showed that mice implanted with cells secreting the class-switch variant 6-19 IgA anti-IgG2a rheumatoid factor, but not 46-42 IgA anti-IgG2a rheumatoid factor, develop glomerular lesions resembling IgA nephropathy. Because the levels of O-linked glycosylation in the hinge region and the structures of N-linked glycans in the CH1 domain differ in 6-19 IgA and 46-42 IgA, we determined the respective contributions of O- and N-linked glycans to the nephritogenic potential of the 6-19 IgA rheumatoid factor in mice. Wild-type 6-19 IgA secreted by implanted cells induced significant formation of glomerular lesions, whereas poorly O-glycosylated 6-19 IgA glycovariants or a 6-19 IgA hinge mutant lacking O-linked glycans did not. However, we observed no apparent heterogeneity in the structure of N-linked glycans attached to three different sites of the Fc regions of nephritogenic and non-nephritogenic 6-19 IgAs. Collectively, our data suggest a critical role of O-linked glycans attached to the hinge region in the development of IgA nephropathy–like GN induced by 6-19 IgA rheumatoid factor in mice.


IgA nephropathy (IgAN) is the most common form of GN worldwide and progresses to end stage renal failure in almost one third of patients with IgAN.1 The glomerular lesions are characterized by mesangial cell proliferation and expansion of the extracellular matrix in association with immune deposits of IgA, which are usually accompanied by deposition of the C3 component of complement and variable codeposition of IgG and IgM. Most significantly, mesangial immune deposits are only observed with the IgA1, but not the IgA2, subclass.7 IgA1 differs from IgA2 through the presence of O-linked glycosylation sites in the hinge region,2 and biochemical analysis of O-linked glycans (O-glycans) of IgA1 in patients with IgAN revealed an aberrant glycosylation status characterized by diminished galactosylation.4–7 Therefore, it has been suggested that aberrant glycosylation of O-glycans of IgA1 is one of the key pathogenic factors contributing to the development of IgAN.

The relative importance of and precise pathogenic mechanism contributed by structural aberrations of O-glycans of IgA1 remain incompletely understood. This was, in part, due to the long-standing belief that O-glycans were absent from the hinge region of murine IgA,8,9 despite the presence of a potential O-linked glycosylation site in murine IgA bearing the Igh-2α and Igh-2c

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allotypes. Indeed, we recently demonstrated the presence of O-glycans in the hinge region of murine IgA monoclonal rheumatoid factors (RFs) bearing the Igh-2z allotype. More significantly, comparative analysis of two murine IgA anti-IgG2a RFs bearing the Igh-2z allele (6-19 and 46-42) revealed that highly O-glycosylated 6-19 IgA RF mAbs induced severe glomerular lesions characterized by abundant mesangial IgA deposits, together with IgG2a and C3, whereas poorly O-glycosylated 46-42 IgA failed to provoke glomerular lesions. However, we also noted a marked difference in the structure of N-linked glycans (N-glycans) present in the CH1 domain between nephritogenic 6-19 IgA RF and non-nephritogenic 46-42 IgA RF (i.e., biantennary and triantennary complex types in 6-19 IgA versus hybrid type with features of both high mannose–type and complex-type oligosaccharides in 46-42 IgA). Thus, we could not exclude the implication of structural differences in N-glycans in IgAN-like glomerular lesions induced by 6-19 IgA RF.

Using the 6-19 model of IgAN-like GN, we aimed to define the contribution of O- and N-glycans to the nephritogenic potential of murine IgA anti-IgG2a RF. To this end, we generated a 6-19 IgA RF mutant that lacks the O-glycosylation acceptor site in the hinge region. In addition, three independent 6-19 IgA anti-IgG2a RF mAbs were established from C57BL/6 (B6) mice constitutively expressing the 6-19 IgA RF transgene (Tg) in order to obtain 6-19 IgA RF glycovariants bearing O-glycans, the extent and composition of which were different from those of the wild-type (WT) 6-19 IgA RF mAb. The analysis of these different 6-19 IgA RF variants compared with the WT 6-19 IgA RF mAb indicates that the extent of O-glycosylation, even if the glycans are highly galactosylated, is an important factor determining the nephritogenic activity of 6-19 IgA RF mAbs.

RESULTS

Structures of N-Glycans of a 6-19 IgA Hinge Mutant Lacking O-Glycans

The 6-19 IgA anti-IgG2a RF mAb induces IgAN-like glomerular lesions characterized by segmental expansion of the mesangial cell matrix and mesangial cell proliferation in association with massive deposits of IgA, IgG2a, and C3 in the mesangium. Because nephritogenic 6-19 IgA RF carries O-glycans in the hinge region at much higher levels than non-nephritogenic 46-42 IgA RF, a 6-19 IgA mutant lacking O-glycans in the hinge region was generated. Because the first threonine (T) residue at position 228 in the hinge region (PTPPPITTIPCSC) of 6-19 IgA bearing the Igh-2z allele is the O-glycosylation acceptor site (Supplemental Figure 1), it was replaced by a proline (PPPPPPPI-TIPSC) to eliminate the O-glycosylation site. The absence of O-glycans in the hinge region of the 6-19 T228P mutant was confirmed by the analysis of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of the HPLC fractions containing the 62-amino acid hinge peptide (generated by treatment with trypsin and lysylendopeptidase). This analysis revealed the presence of a single peak containing the nonglycosylated hinge peptide at a mass to charge ratio (m/z) of 6627, with no concomitant glycosylated hinge peptides (Figure 1).

In addition, we compared the structure of N-glycans present at asparagine position 162 (N162) in the CH1 domain, at N438 in the CH3 domain, and at N453 in the secretary tailpiece of 6-19 T228P hinge mutant with those of WT 6-19 IgA (Supplemental Figure 1). MALDI-TOF MS analysis showed that the CH1 domain of both 6-19 IgA mAbs carried biantennary and triantennary complex-type oligosaccharide chains at N162 (Figure 2). Although we noted that a fraction of biantennary complex-type glycans of the 6-19 T228P mutant were devoid of galactose residues (glycopeptide at m/z 5733), the majority of them terminated with galactose plus sialic acid (N-glycolyneuraminic acids) residues, as was the case with WT 6-19 IgA (Figure 2). In contrast, the glycoforms attached to N438 and N453 of the 6-19 IgAT228P mutant were essentially identical to those of WT 6-19 IgA: N-glycans at position 438 had a mixed composition of high mannose– and complex-type (biantennary and triantennary) oligosaccharide chains (Figure 3) and those at position 453 were exclusively of high mannose type (Figure 4).

Lack of Induction of Glomerular Lesions by a 6-19 IgA Hinge Mutant Lacking O-Glycans

We previously showed that BALB/c mice implanted with transfectoma cells secreting WT 6-19 IgA RF developed severe glomerular lesions resembling human IgAN. Therefore, the nephritogenic potential of the 6-19 IgA T228P hinge mutant was assessed by implantation of transfectoma cells secreting the 6-19 T228P mutant into BALB/c mice. Three weeks after intraperitoneal injection of 6-19 T228P transfectoma cells, serum concentrations of IgA anti-IgG2a RF and IgA-IgG2a immune complexes (ICs) were markedly increased at levels even higher than those seen in mice implanted with cells secreting WT 6-19 IgA (Table 1). Despite high serum concentrations of 6-19 IgA RF and IgA-IgG2a IC, no substantial histologic alterations were observed in glomeruli of mice implanted with transfectoma cells secreting 6-19 T228P (Figure 5, Table 1). Immunofluorescence analysis revealed only minimal deposits of IgA, IgG2a, and C3. Notably, serum concentrations of 6-19 IgA RF in mice implanted with T228P- or WT 6-19–secreting cells reflected those of the polymeric form, because Sephadex G200 gel filtration chromatography of the purified 6-19 T228P mutant showed that only its polymeric form displayed anti-IgG2a RF activities (data not shown), as observed with WT 6-19 IgA.

Structures of O- and N-Glycans of 6-19 IgA mAbs Derived from 6-19 IgA Tg Mice

The relevance of the results obtained with the 6-19 T228P hinge mutant lacking O-glycans could be dismissed by arguing that the replacement of threonine by proline in the hinge region might induce conformational changes in 6-19 IgA molecules, thereby affecting their glomerular localization. We, therefore, generated additional 6-19 IgA glycovariants with heavy and light chains that were identical to WT 6-19 IgA, but poorly O-glycosylated,
because of their synthesis by alternative hybridoma cells. To this end, a panel of IgA anti-IgG2a RF mAb was established from 6-19 IgA Tg B6 mice, which spontaneously produce 6-19 IgA anti-IgG2a RF. We selected three independent clones, designated 6-19 Tg-1, Tg-2, and Tg-3, which secreted high concentrations of IgA RF in vitro. Nucleotide sequence analyses confirmed that the amino acid sequences of heavy and light chains of these three mAbs are identical to those of WT 6-19 IgA RF mAbs. Again, as expected, Sephadex G200 gel filtration chromatography indicated that anti-IgG2a RF activities of 6-19 Tg IgAs were only detectable in fractions containing the polymeric form of IgA (data not shown).

MALDI-TOF MS analyses revealed that the hinge region of the three different 6-19 Tg IgA mAbs carried O-glycans, the carbohydrate composition of which was either N-acetylgalactosamine-galactose disaccharide or its sialylated forms, as in the case of WT 6-19 IgA (Figure 1, Supplemental Figure 2). However, the dominance of a peak containing the nonglycosylated hinge peptide at m/z 6627 over those containing the glycosylated peptides indicated that only a limited fraction of these three IgA mAbs was O-glycosylated compared with WT 6-19 IgA. Notably, the extent of O-glycosylation of the three different 6-19 Tg IgAs was comparable to that of the non-nephritogenic 46-42 IgA mAb11. In contrast, MALDI-TOF MS profiles of respective glycoforms attached at N162, N438, and N453 of three 6-19 Tg IgAs were comparable to those observed with WT 6-19 IgA, except for N-glycans at N453 of Tg-3 IgA, which displayed a mixed composition of high mannose– and biantennary complex-type oligosaccharide chains lacking galactose residues, whereas those of the two other 6-19 Tg IgAs were exclusively of high mannose type, such as those of WT 6-19 IgA (Figures 2–4, Supplemental Figures 3–5).

**Figure 1.** Linear MALDI-TOF mass spectra of glycopeptides bearing O-glycans from the hinge region of 6-19 IgA and its glycovariants. Note differences in the extent of O-glycosylation between 6-19 WT and Tg-1 IgAs and the absence of O-glycans in 6-19 T228P mutant. The glycoforms are indicated above the corresponding glycopeptide peaks (□, N-acetylgalactosamine; ○, galactose; ◇, N-glycolyneuraminic acid). Peaks without indication of glycoforms are derived from other peptides.

Lack of Induction of Glomerular Lesions by 6-19 IgA mAbs Derived from 6-19 IgA Tg Mice

The nephritogenic activity of 6-19 Tg IgA mAbs was assessed by implantation of hybridoma cells secreting each 6-19 Tg IgA mAb into BALB/c mice. As in the case of mice implanted with 6-19 WT or T228P transfectedoma cells, intraperitoneal implantation of 6-19 IgATg hybridoma cells induced within 3 weeks increases in serum IgA anti-IgG2a RF and IgA-IgG2a IC at levels higher than, or comparable to, those of mice implanted with WT 6-19 IgA–secreting cells (Table 1). Again, mice implanted with either of the three different 6-19 Tg IgA hybridoma cells failed to develop significant glomerular...
lesions, and these glomeruli displayed only minimal deposits of IgA, IgG2a, and C3 (Figure 5, Table 1). Because the structural analysis of O- and N-glycans of 6-19 IgA mAbs was carried out on those purified from the culture supernatant of hybridomas in vitro, one cannot exclude the possibility that the extent and pattern of O- and N-glycosylations of 6-19 IgA mAbs could be different in the in vivo environment. However, the analysis of 6-19 WT and Tg IgA mAbs purified from ascites confirmed that the glycosylation status of 6-19 IgAs in vivo was essentially identical to that observed in vitro (Supplemental Figures 6 and 7).

**DISCUSSION**

This study was designed to assess the nephritogenic potential of different 6-19 IgA anti-IgG2a RF glycovariants in relation to the abundance of O-glycans in the hinge region and the structural differences of N-glycans attached to three different sites of the Fc region of IgA. We demonstrate that in contrast to a potent nephritogenic activity of the WT 6-19 IgA mAb, which was highly O-glycosylated, 6-19 T228P hinge mutants lacking O-glycans as well as poorly O-glycosylated 6-19 IgA variants newly established from 6-19 IgA Tg mice are unable to induce significant glomerular lesions. In addition, we observed that structures of N-glycans attached to the CH1 and CH3 domains and the secretary tailpiece were essentially identical between nephritogenic and non-nephritogenic 6-19 IgAs. These data argued in favor of a major role of O-glycans in the pathogenesis of IgAN-like glomerular lesions induced by the 6-19 anti-IgG2a RF mAb.

We previously showed that 46-42 IgA anti-IgG2a RF failed to induce glomerular lesions, despite the fact that this mAb has the same IgA allotype as 6-19 IgA RF and that relative concentrations of polymeric versus monomeric forms of 46-42 IgA RF were comparable to those of 6-19 IgA. Although our previous

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**Figure 2.** Linear MALDI-TOF mass spectra of glycopeptides bearing N-glycans attached to N162 in the CH1 domain of 6-19 IgA and its glycovariants. Note that the majority of the N-glycans of 6-19 WT, T228P, and Tg-1 IgAs are terminated with galactose plus N-glycolyneuraminic acid residues, whereas a fraction of biantennary glycans are devoid of galactose (m/z 5733). The glycoforms are indicated above corresponding glycopeptide peaks (●, N-acetylglucosamine; gray circle, mannose; ○, galactose; ◇, N-glycolyneuraminic acid; dark gray triangle, fucose). Peaks without indications of glycoforms are derived from other peptides.
The present analysis of different 6-19 IgA glycovariants compared with WT 6-19 IgA argued against this possibility, because non-nephritogenic 6-19 IgA variants carry variable regions’ sequences identical to those of WT 6-19 IgA RF in both heavy and light chains. Notably, serum levels of IgA RF and IgA-IgG2a IC in mice implanted with cells secreting different 6-19 IgA variants were similar to those of mice implanted with WT 6-19 IgA–secreting cells.

Accordingly, the present results support the idea that the distinct nephritogenic potential of the 6-19 versus 46-42 IgA RF mAb can be attributed to the difference observed in the abundance of O-glycans attached to the hinge region of IgA. Indeed, only the highly O-glycosylated 6-19 IgA RF displayed potent nephritogenic activity, whereas 6-19 IgA variants that were either lacking O-glycans or were poorly O-glycosylated in the hinge region at levels comparable to non-nephritogenic 46-42 IgA failed to induce significant glomerular lesions. Moreover, the lack of nephritogenic potential of the 6-19 Tg IgA mAbs tested is consistent with our preliminary findings that 6-19 IgA Tg mice failed to develop IgAN-like glomerular lesions, despite the constitutive production of substantial amounts of 6-19 IgA.

The development of IgAN in humans is associated with structural aberrations of O-linked glycans in the IgA1 hinge region. Because galactose-deficient variants are predominantly found in circulating ICs and glomerular immune deposits in patients with IgAN, it has been suggested that hypogalactosylation of the IgA1 O-glycans and the subsequent development of IgA1 ICs are critically involved in the pathogenesis of IgAN. Indeed, studies revealed that N-acetylgalactosamine exposed on the galactosylated O-glycans represents the major component of the neoepitope that is recognized by naturally occurring IgG antibodies. Consequently, IgA1-IgG ICs composed of galactose-deficient IgA1 as an antigen and N-acetylgalactosamine–specific IgG as an antibody are formed and tend to be localized in the mesangium, thereby inducing glomerular injury. However, it should be stressed that nephritogenic murine 6-19 IgA O-glycans are highly galactosylated and that the development of 6-19 IgA RF–induced glomerular lesions is dependent on the formation of the present study was hampered by the fact that the possible differences in the fine specificity and affinity of anti-IgG2a RF activity between these two RF mAbs could influence their nephritogenic potential.

Figure 3. Linear MALDI-TOF mass spectra of glycopeptides bearing N-glycans attached to N438 in the CH3 domain of 6-19 IgA and its glycovariants. Note the presence of both high mannose-type and complex-type glycoforms in 6-19 WT, T228P, and Tg-1 IgAs. The glycoforms are indicated above corresponding glycopeptide peaks. M5 and M6 indicate the high mannose–type oligosaccharides containing five and six mannose residues, respectively. Peaks without indications of glycoforms are derived from other peptides.
of IgA RF-IgG2a ICs, as previously described. Clearly, the implication of O-glycans in the development of IgAN in humans and the 6-19 IgA RF model of GN is different. Nevertheless, one cannot exclude the possibility that IgA RF-IgG ICs could play a role in the pathogenesis of IgAN in some patients, because increases in serum levels of IgA RF have been reported in approximately 50% of patients with IgAN. It is also worth mentioning that nine potential O-glycosylation sites are present in the hinge region of human IgA1 and that the attachment of as many as six O-linked oligosaccharide side chains has been identified. Notably, the variability in the distribution and density of O-glycan chains at each site and in the composition of O-glycan structures of each chain creates remarkable site-specific and compositional heterogeneity of O-glycans of IgA1. This is a marked contrast to the presence of only one O-glycosylation acceptor site and, hence, a limited heterogeneity of O-glycans in murine IgA bearing the IgH-2a or IgH-2c allotype. Thus, results obtained in murine models of IgAN cannot be directly extrapolated to understand the contribution of aberrant O-glycosylation to the pathogenesis of human IgAN. In this regard, it would be of interest to generate 6-19 and 46-42 IgA variants bearing the hinge sequence of human IgA1 and evaluate their nephritogenic potential in relation to the abundance and structure of their O-glycans.

Our previous analysis of 6-19 and 46-42 IgAs also revealed marked differences in the structure of N-glycans attached at N162 in the CH1 domain: complex type in 6-19 IgA versus hybrid type in 46-42 IgA. However, structures of N-glycans at N162 in the 6-19 T228P hinge mutant, as well as three independent 6-19 IgA mAbs established from 6-19 IgA Tg mice, were similar to those of the pathogenic WT 6-19 IgA mAb. These data argue against a primary role of N-glycans present in the Fc region of IgA for the development of IgAN-like glomerular lesions induced by the 6-19 IgA RF mAb. However, because it was previously reported that hypogalactosylation of N-glycans of serum IgA was associated with the development of glomerular lesions in other murine models of IgAN, one cannot exclude the possibility that aberrant glycosylation of N-glycans could play an additional role in the development of IgAN-like glomerular lesions. In this regard, it should be stressed that the development of IgAN-like glomerular lesions has been reported in ddY-A mice bearing the IgH-2b allotype, in which IgA lacks the O-glycosylation acceptor site, whereas a more rapid and severe disease was developed in ddY-B mice, in which IgA are potentially O-glycosylated.

Figure 4. Linear MALDI-TOF mass spectra of glycopeptides bearing N-glycans attached to N453 in the secretary tailpiece of 6-19 IgA and its glycovariants. Note the presence of only high mannose type in WT, T228P, and Tg-1 IgA. The glycoforms are indicated above corresponding glycopeptide peaks. Peaks without indication of glycoforms are derived from other peptides.
The generation of the WT 6-19 IgA anti-IgG2a RF mAb was previously described.11 The 6-19 IgA T228P mutant was generated by transfecting a 6-19 heavy chain-loss cell line with the VDJH6-19-Cκ plasmid containing the complete 6-19 IgA heavy and light chain genes into fertilized eggs of B6 mice. Mice were screened for the 6-19 IgA Tg by determining serum levels of IgA by ELISA and confirmed by PCR analysis. A founder expressing the 6-19 IgA Tg, which was stably inherited, has been established and is used in this study. Animal experiments described in this study were approved by the Ethics Committee for Animal Experimentation of the University of Geneva.

**CONCISE METHODS**

**Mice**

BALB/c and B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The VDJH6-19-Cα-VJκ6-19-Cκ plasmid containing the complete 6-19 IgA heavy chain and light chain genes was microinjected into fertilized eggs of B6 mice. Mice were screened for the 6-19 IgA Tg by determining serum levels of IgA by ELISA and confirmed by PCR analysis. A founder expressing the 6-19 IgA Tg, which was stably inherited, has been established and is used in this study. Animal experiments described in this study were approved by the Ethics Committee for Animal Experimentation of the University of Geneva.

**DNA Construction**

The construction of the VDJH6-19-Cα plasmid containing the complete 6-19 heavy chain gene of the IgA class was previously described.11 The VDJH6-19-Cα(T228P) plasmid bearing a mutation at position 228 (threonine to proline) was generated by oligonucleotide-directed mutagenesis. The VDJH6-19-Cα3-VJκ6-19-Cκ plasmid containing the complete 6-19 IgA heavy and light chain genes was constructed using the following DNA fragments: cDNA encoding the variable region of the heavy and light chains of the 6-19 mAb,24 the promoter region from the heavy chain enhancer region from pSVE2-neo,26 the Cκ region from the genomic clone plgμ-8 isolated from BALB/c mice,27 and the Cκ region derived from pEVH-Cκ-neo (a kind gift from Dr. K. Rajewsky, Institute for Genetics, University of Cologne, Cologne, Germany).

**Table 1.** Serum levels of IgA anti-IgG2a RF and IgA-IgG2a IC, and the development of glomerular lesions in BALB/c mice implanted with cells secreting 6-19 IgA or its glyvairants

<table>
<thead>
<tr>
<th>6-19 IgA</th>
<th>IgA Anti-IgG2a (mg/ml)</th>
<th>IgA-IgG2a IC (OD)</th>
<th>Glomerular Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.45 ± 0.03</td>
<td>0.38 ± 0.04</td>
<td>10/10</td>
</tr>
<tr>
<td>T228P</td>
<td>0.88 ± 0.15</td>
<td>0.51 ± 0.05</td>
<td>0/12</td>
</tr>
<tr>
<td>Tg-1</td>
<td>0.90 ± 0.16</td>
<td>0.54 ± 0.08</td>
<td>0/8</td>
</tr>
<tr>
<td>Tg-2</td>
<td>0.60 ± 0.10</td>
<td>NT</td>
<td>0/7</td>
</tr>
<tr>
<td>Tg-3</td>
<td>0.53 ± 0.09</td>
<td>NT</td>
<td>0/7</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM, unless otherwise indicated. NT, not tested.

*a* Serum levels of IgA anti-IgG2a RF 3 weeks after implantation of 6-19 IgA RF-secreting cells in 2- to 3-month-old BALB/c mice (n=7–12 mice). Serum levels of IgA anti-IgG2a before injection of cells were <0.01 mg/ml.

*b* Serum levels of IgA-IgG2a IC 3 weeks after implantation of 6-19 IgA RF-secreting cells, expressed as OD at 405 nm (n=8–12 mice). Serum levels of IgA-IgG2a IC before injection of cells were 0.07 ± 0.004.

*c* Incidence of glomerular lesions evaluated by histologic examination.

Table 1. Serum levels of IgA anti-IgG2a RF and IgA-IgG2a IC, and the development of glomerular lesions in BALB/c mice implanted with cells secreting 6-19 IgA or its glyvairants

Clearly, thorough analyses of the structures and extent of oligosaccharide side chains in these two ddY sublines should help define the respective contributions of O- and N-glycans to IgAN pathogenesis in the ddY model.

This study provides evidence supporting the critical role of O-glycans in the IgA hinge region for the development of IgAN-like GN induced by 6-19 IgA RF. However, how O-glycans present in the 6-19 IgA hinge region are implicated in the glomerular localization of IgA and subsequent development of glomerular inflammation remains to be defined, although it can be speculated that a conformational change in the Fc region due to the presence of O-glycans could promote nephritogenicity. Moreover, it is of importance to define the molecular mechanism involved in the regulation of O-glycosylation because the extent of occupancy and localization of O-glycans on the potential O-glycosylation sites are highly variable among individual human IgA1 and murine IgA. In this regard, it is worth noting that a study reported the possible role of T-cell cytokine IL-4 in the regulation of O-glycosylation of the IgA1 hinge region. Further understanding of the interplay between the structural characteristics of O-glycosylated IgA and their nephritogenic properties, as well as the molecular basis responsible for the regulation of IgA O-glycosylation, could help not only to improve our understanding of the immunopathologic mechanisms central to the development of IgAN but also to develop new therapeutic approaches for this disease.

**Figure 5.** Representative histologic appearance and immune deposits in BALB/c mice implanted with transfectedoma or hybridoma cells secreting 6-19 WT, T228P hinge mutant, or Tg IgA mAb. Note segmental expansion of mesangial matrix and focal proliferation of mesangial cells in mice implanted with WT 6-19 IgA-secreted cells, but essentially normal histologic appearance of glomeruli in mice implanted with cells secreting 6-19 T228P or Tg-1 IgA (periodic acid–Schiff staining). Immunohistochemical analysis reveals extensive fine granular deposits of IgA, IgG2a, and C3 in the mesangium of mice implanted with WT 6-19 IgA-secreting cells, but only minimal extent of these deposits in mice implanted with cells secreting 6-19 T228P or Tg-1 IgA. Because similar results are obtained with the two other 6-19 Tg IgA mAbs, only results obtained with 6-19 Tg-1 IgA are shown. Original magnification, ×400.
IgG2a RF mAbs bearing the heavy and light chains identical to WT 6-19 IgA were established from 6-19 IgATg B6 mice bearing the Igk⁺ allotype. IgA mAbs were purified from culture supernatants or ascites by an affinity column coupled with 11.44 rat anti-mouse IgA mAb, a kind gift of Dr. John Kearney (University of Alabama, Birmingham, AL), or anti-6-19 idiotypic mAb.²⁸ The purity was >95% as documented by SDS-PAGE.

**Implantation of Transfectoma or Hybridoma Cells**

To study the nephritogenicity of 6-19 IgA anti-IgG2a RF mAbs, 10⁷ transfectoma or hybridoma cells secreting 6-19 IgA anti-IgG2a RF mAb were injected intraperitoneally into pristine-treated BALB/c mice that were euthanized when moribund. To avoid rejection of the transfectoma or hybridoma cells, immunosuppression was achieved by a simultaneous injection of a mixture of anti-mouse CD4 (GK1.5) and anti-mouse CD8 (H-35) mAb (0.5 mg of each mAb), as previously described.²⁹ Kidneys were obtained at autopsy, processed for histologic examination, and stained with periodic acid–Schiff. Glomerular deposition of IgA and IgG2a was determined by its were examined by direct staining with goat anti-mouse C3 conjugates (Vector Laboratories, Inc., Burlingame, CA). C3 deposits were quantified using a Sephadex G200 gel filtration column coupled with 11.44 rat anti-mouse IgA mAb, a kind gift of Dr. John Kearney, as previously described.²⁸ The purity was >95% as documented by SDS-PAGE.

Serologic Assays

Serum levels of IgA anti-IgG2a RF were determined by ELISA, as previously described.¹巴 Briefly, microtiter plates were coated with TNP8-BSA and subsequently incubated with Hy1.2 IgG2a anti-TNP mAb before the addition of serum samples. The assay was developed with alkaline phosphatase–labeled 11.44 rat anti-mouse IgA mAb. Results are expressed as milligrams per milliliter of IgA anti-IgG2a by referring to a standard curve obtained from purified 6-19 IgA anti-IgG2a RF mAb. Serum concentrations of IgA-IgG2a ICs were quantified by ELISA, in combination with precipitation of serum by polyethylene glycol (Siegfried, Zollikon, Switzerland), which allowed only precipitation of IgA RF-IgG2a ICs but not free IgG2a. The precipitates were solubilized in PBS containing 1% BSA and 0.05% Tween-20 and subjected to ELISA using plates coated with goat anti-mouse IgA (Bethyl Laboratories, Inc., Cambridge, UK) and the assay was developed with alkaline phosphatase–labeled goat anti-mouse IgG2a conjugates (Cappel Laboratories, West Chester, PA).

Gel Filtration

The size distribution of 6-19 IgA anti-IgG2a RF mAb was analyzed using a Sephadex G200 gel filtration column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, and elution was performed using the same buffer at a flow rate of 0.2 ml/min at 4°C. The column was calibrated with blue dextran (void volume), IgG (150 kD), and BSA (67 kD). Relative concentrations of IgA and IgA anti-IgG2a RF in each fraction were estimated by ELISA.

RT-PCR and cDNA Sequencing

RNA from 6-19 IgA hybridoma cells established from 6-19 IgATg mice was purified with TRIzol reagent (Invitrogen AG, Basel, Switzerland). For nucleotide sequencing of the entire variable and constant regions of the heavy and light chains of 6-19 IgA mAbs, cDNA was amplified with Pfu DNA polymerase (Stratagene Cloning Systems, La Jolla, CA) using the following pairs of primers: VH1BACK forward primer³² and 3’ untranslated Ca reverse primer for the heavy chain; VK1BACK forward primer³² and Ck reverse primer for the light chain, as described.¹¹

**Analyses of Oligosaccharide Structures**

The oligosaccharide profiles were analyzed by linear MALDI-TOF MS of glycopeptides according to a previously described method.¹²,²² Briefly, 6-19 IgA mAbs were reduced by dithiothreitol and carboxamidomethylated with iodoacetamide in a solution of 6 M guanidinium chloride, 0.25 M Tris-HCl, and 1 mM EDTA (pH 8.0). The alkylated proteins were desalted with a NAP-5 gel filtration column (GE Healthcare, Buckinghamshire, UK) equilibrated with 0.05 M HCl. The pH was then adjusted to 8.5 by titration with a 1.5 M Tris solution for digestion, which was performed with a mixture of trypsin (Promega, Buckinghamshire, UK) and lysylendopeptidase (Wako, Osaka, Japan). Peptides in the digest were separated by HPLC using a 1.0 mm×150 mm C8 reversed phase column (Inertsil WP300; GL Science, Tokyo, Japan) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Identification and characterization of glycopeptides in the elution fractions were carried out by MALDI MS using a Voyager DE Pro time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA), which was operated in the linear mode. The sample matrix was 2,5-dihydroxybenzoic acid, which was dissolved in 50% acetonitrile at a concentration of 20 mg/ml. An aliquot of an HPLC fraction was mixed with a dihydroxybenzoic acid solution on the MALDI sample target.

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**DISCLOSURES**

None.

**REFERENCES**


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Figure S1. O- and N-glycosylation sites in the Fc region of 6-19 IgA mAb bearing the \textit{Igh}-2\textsuperscript{a} allotype. The 62 amino-acid hinge peptide yielded by treatment with trypsin and lysylendopeptidase is underlined, with the hinge region highlighted in blue. The O-linked glycosylation site at threonine position 228 and three N-linked glycosylation sites at N162, N438 and N453 are boxed.
**Figure S2.** MALDI linear-TOF mass spectra of glycopeptides bearing O-glycans from the hinge region of 6-19 Tg-2 and Tg-3 IgA mAbs. The glycoforms are indicated above the corresponding glycopeptide peaks (open square: N-acetylgalactosamine; open circle: galactose; open diamond: N-glycolylneuraminic acid). Peaks without indication of glycoforms were derived from other peptides. *m/z*: mass-to-charge ratio.
Figure S3. MALDI linear-TOF mass spectra of glycopeptides bearing N-glycans attached to N162 in the CH1 domain of 6-19 Tg-2 and Tg-3 IgA mAbs. The glycoforms are indicated above corresponding glycopeptide peaks (closed square: N-acetylglucosamine; gray circle: mannose; open circle: galactose; open diamond: N-glycolylneuraminic acid; dark gray triangle: fucose). Peaks without indications of glycoforms were derived from other peptides.
**Figure S4.** MALDI linear-TOF mass spectra of glycopeptides bearing N-glycans attached to N438 in the CH3 domain of 6-19 Tg-2 and Tg-3 IgA mAbs. The glycoforms are indicated above corresponding glycopeptide peaks. M5 and M6 indicate the high mannose-type oligosaccharides containing five and six mannose residues, respectively. Peaks without indications of glycoforms were derived from other peptides.
Figure S5. MALDI linear-TOF mass spectra of glycopeptides bearing N-glycans attached to N453 in the secretary tailpiece of 6-19 Tg-2 and Tg-3 IgA mAbs. Note the presence of high mannose-type and complex-type glycoforms in Tg-3 IgA, but the presence of only high mannose-type in Tg-2 IgA. The glycoforms are indicated above corresponding glycopeptide peaks, and peaks without indication of glycoforms were derived from other peptides.
**Figure S6.** MALDI linear-TOF mass spectra of glycopeptides bearing O-glycans in the hinge region (O228) or N-glycans in the CH1 (N162) and CH3 (N438) domains and the secretary tailpiece (N453) of WT 6-19 IgA purified from culture supernatant (left panel) or from ascites of mice implanted with WT 6-19 IgA hybridoma (right panel). Note that the glycosylation patterns of O- and N-glycans in the *in vitro* and *in vivo* settings were comparable. The glycoforms are indicated above the corresponding glycopeptide peaks, and peaks without indication of glycoforms were derived from other peptides.
Figure S7. MALDI linear-TOF mass spectra of glycopeptides bearing O-glycans in the hinge region (O228) or N-glycans in the CH1 (N162) and CH3 (N438) domains and the secretary tailpiece (N453) of 6-19 Tg-1 IgA purified from culture supernatant (left panel) or from ascites of mice implanted with 6-19 Tg-1 IgA hybridoma (right panel). As observed with 6-19 WT IgA, the glycosylation patterns of O- and N-glycans in the in vitro and in vivo settings were comparable. Notably, this was also the case of Tg-2 and Tg-3 IgAs (data not shown). The glycoforms are indicated above the corresponding glycopeptide peaks, and peaks without indication of glycoforms were derived from other peptides.