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*J Clin Invest.* 1999;104(1):73-81. <https://doi.org/10.1172/JCI5535>.

### Article

Circulating immune complexes (CICs) isolated from sera of patients with IgA nephropathy (IgAN) consist of undergalactosylated, mostly polymeric, and J chain-containing IgA1 and IgG antibodies specific for *N*-acetylgalactosamine (GalNAc) residues in *O*-linked glycans of the hinge region of IgA1 heavy chains. Antibodies with such specificity occur in sera of IgAN patients, and in smaller quantities in patients with non-IgA proliferative glomerulonephritis and in healthy controls; they are present mainly in the IgG (predominantly IgG2 subclass), and less frequently in the IgA1 isotype. Their specificity for GalNAc was determined by reactivity with IgA1 myeloma proteins with enzymatically removed *N*-acetylneuraminic acid (NeuNAc) and galactose (Gal); removal of the *O*-linked glycans of IgA1 resulted in significantly decreased reactivity. Furthermore, IgA2 proteins that lack the hinge region with *O*-linked glycans but are otherwise structurally similar to IgA1 did not react with IgG or IgA1 antibodies. The re-formation of isolated and acid-dissociated CICs was inhibited more effectively by IgA1 lacking NeuNAc and Gal than by intact IgA1. Immobilized GalNAc and asialo-ovine submaxillary mucin (rich in *O*-linked glycans) were also effective inhibitors. Our results suggest that the deficiency of Gal in the hinge region of IgA1 molecules results in the generation of antigenic determinants containing GalNAc residues that are recognized by naturally occurring IgG and IgA1 antibodies.

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# Circulating immune complexes in IgA nephropathy consist of IgA1 with galactose-deficient hinge region and antiglycan antibodies

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Received for publication October 16, 1998, and accepted in revised form May 20, 1999.

Circulating immune complexes (CICs) isolated from sera of patients with IgA nephropathy (IgAN) consist of undergalactosylated, mostly polymeric, and J chain-containing IgA1 and IgG antibodies specific for *N*-acetylgalactosamine (GalNAc) residues in *O*-linked glycans of the hinge region of IgA1 heavy chains. Antibodies with such specificity occur in sera of IgAN patients, and in smaller quantities in patients with non-IgA proliferative glomerulonephritis and in healthy controls; they are present mainly in the IgG (predominantly IgG2 subclass), and less frequently in the IgA1 isotype. Their specificity for GalNAc was determined by reactivity with IgA1 myeloma proteins with enzymatically removed *N*-acetylneuraminic acid (NeuNAc) and galactose (Gal); removal of the *O*-linked glycans of IgA1 resulted in significantly decreased reactivity. Furthermore, IgA2 proteins that lack the hinge region with *O*-linked glycans but are otherwise structurally similar to IgA1 did not react with IgG or IgA1 antibodies. The re-formation of isolated and acid-dissociated CICs was inhibited more effectively by IgA1 lacking NeuNAc and Gal than by intact IgA1. Immobilized GalNAc and asialo-ovine submaxillary mucin (rich in *O*-linked glycans) were also effective inhibitors. Our results suggest that the deficiency of Gal in the hinge region of IgA1 molecules results in the generation of antigenic determinants containing GalNAc residues that are recognized by naturally occurring IgG and IgA1 antibodies.

*J. Clin. Invest.* 104:73–81 (1999).

## Introduction

IgA nephropathy (IgAN) is the most common glomerulonephritis worldwide (1–3). Clinically, it is characterized by hematuria and proteinuria; about 20–30% of the IgAN patients develop progressive renal failure within 10–20 years from the onset of disease (1, 3). Histologically, the glomerular mesangium contains deposits of IgA1, the C3 component of complement, and less frequently, IgG and/or IgM (4). Circulating immune complexes (CICs) composed of IgA1, C3, and IgG (5) are thought to be involved in the pathogenesis of the disease (3, 6, 7). However, the nature of the potential antigen(s) in CICs and mesangial deposits has remained unknown (8).

Recent analyses of serum IgA1 from IgAN patients revealed significant alterations in the glycan side chains (9–17). In contrast to IgG, IgM, and IgA2, human IgA1 contains *N*- and *O*-linked glycans; the latter are bound to seryl and threonyl residues in the unique hinge region of this isotype (18–20). In general, it is agreed that IgA1 molecules from IgAN patients

display altered glycan moieties, usually with a reduced content of galactose (Gal) (9–17). Recently, we have demonstrated that the Gal-deficient IgA1 is present in CICs with IgG; no significant difference was detected in the content of Gal in uncomplexed serum IgA1 from IgAN patients or controls (9). Increased binding of IgA1 from sera of IgAN patients to lectins specific for a terminal GalNAc, such as *Helix aspersa* (HAA) or *Helix pomatia* (HPO), indicated that the aberrant glycans were located in the hinge region of IgA1 proteins (9, 10). In addition to a deficiency of Gal residues, the hinge region glycans of IgA1 from IgAN patients also differ from those of controls in another aspect. They contain an increased number of *N*-acetylneuraminic acid (NeuNAc) residues attached to GalNAc (14), as shown by the increased binding of GalNAc-specific lectins after removal of NeuNAc (9).

Because Gal-deficient IgA1 was present in CICs with IgG (9), we considered the possibility that the interaction of these 2 component immunoglobulin molecules may be based on antigen (IgA1)–antibody (IgG) recog-

nition. Indeed, our earlier studies demonstrated that sera of IgAN patients, as well as those of normal individuals, contain naturally occurring IgG and IgM antibodies that react with the Fab fragment of IgA1, but not IgA2, myeloma proteins (21–23). Fortuitously, we used IgA1 protease from *Haemophilus influenzae*, which cleaves the hinge region of IgA1 heavy chain so that most of the O-linked glycans remained associated with the Fab fragment (24).

The present study was conducted to determine the role of hinge region glycans in interactions of IgA1 with serum IgG, to investigate the molecular property of IgA1-IgG immune complexes, and to examine whether similar interactions also exist between IgA1 with abnormal structure of hinge region glycans and serum IgM or IgA.

## Methods

**Human subjects.** Sera from 88 biopsy-proven IgAN patients (60 men and 28 women, age  $37.5 \pm 14.2$  years), 20 non-IgA mesangial proliferative glomerulonephritis (non-IgA GN) patients (12 men and 8 women, age  $44.3 \pm 13.4$  years), and 91 healthy volunteers of comparable age and sex distribution were collected from volunteers at the University of Alabama–Birmingham Hospital and Clinics; the University of Alabama–Birmingham employees; and the School of Medicine, Palacky University. Informed consent was obtained before collecting samples.

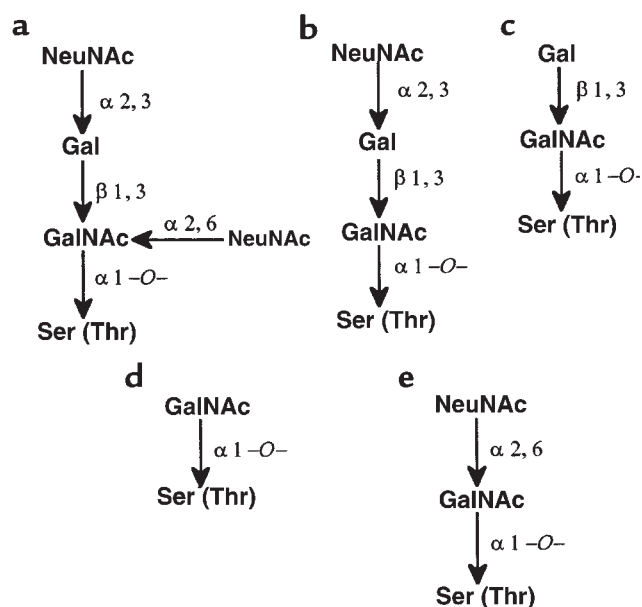
**Isolation of myeloma proteins.** Monomeric (m) and polymeric (p) IgA1 and pIgA2 myeloma proteins were isolated from plasma of patients with IgA multiple myeloma by methods described previously (25), which included ammonium sulfate precipitation, gel filtration, starch-block electrophoresis, affinity chromatography on jacalin (a lectin specific for Gal- $\beta$ 1,3-GalNAc) (26), and immunoabsorption. The Fab fragments of IgA1 myeloma proteins were prepared by cleavage with bacterial IgA1 protease from *H. influenzae* HK50 as described (25) and were further purified by size-exclusion chromatography and affinity chromatography on immobilized concanavalin A. Carbohydrate analyses of 2 Fab preparations (Ste and Ber) revealed only O-linked glycans. Concanavalin A and jacalin conjugated to agarose were purchased from Pierce Chemical Co. (Rockford, Illinois, USA).

**Modification of the carbohydrate moieties of IgA.** NeuNAc residues were removed using neuraminidase from *Vibrio cholerae* (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA). Gal residues linked to GalNAc in the hinge region of IgA1 were cleaved with  $\beta$ -galactosidase from bovine testis (Boehringer Mannheim Biochemicals), which hydrolyzes  $\beta$ 1,3 linkages substantially faster than  $\beta$ 1,4 or  $\beta$ 1,6 linkages (27). O-linked glycans were partially removed by incubation with neuraminidase and endo-O-glycosidase (Boehringer Mannheim Biochemicals). IgA1 preparations degalactosylated or deglycosylated in the hinge region were separated from intact or desialylated IgA1 by affinity chromatography on a column of immobilized jacalin.

Neuraminidase,  $\beta$ -galactosidase, and liberated monosaccharides were separated from IgA by gel filtration on Superdex 200 (Pharmacia Biotech, Piscataway, New Jersey, USA) or by HPLC on TSK-3000 columns (Phenomenex, Torrance, California, USA). The glycan content was tested by gas chromatography and by ELISA with biotinylated lectins such as HAA or jacalin.

**Preparation of CICs.** IgA1-containing CICs were partially purified from sera using Perkin-Elmer Series 10 (Perkin-Elmer Corp., Norwalk, Connecticut, USA) HPLC equipped with a size-exclusion TSK-5000 column ( $300 \times 7.5$  mm; Phenomenex) equilibrated with 0.02 M sodium phosphate buffer (pH 7.5) containing 0.05 M  $\text{Na}_2\text{SO}_4$ , or by low-pressure size-exclusion chromatography using a Superose 6 column ( $600 \times 12$  mm; Pharmacia Biotech). The molecular form (monomer and/or polymer) of complexed IgA1 was determined, after dissociation of CICs at pH 3.0 (5), by HPLC using a TSK-3000 column ( $300 \times 7.5$  mm; Phenomenex) equilibrated with 0.1 M glycine-HCl buffer (pH 3.0). Fractions were neutralized and tested by ELISA for the content of IgM, IgA1, IgG, J chain, and proteins reacting with HAA. Biotinylated polyclonal antibodies to IgM and F(ab')<sub>2</sub> fragment of anti-IgA and anti-IgG (all heavy chain specific) were purchased from Southern Biotechnology Associates Inc. (Birmingham, Alabama, USA). The biotinylated HAA was purchased from EY Laboratories (San Mateo, California, USA) and Sigma Chemical Co. (St. Louis, Missouri, USA). The assay for J chain (indicating pIgA) in IgA-containing fractions was conducted as described (28).

**Inhibition studies.** IgA1-IgG CICs prepared by low-pressure size-exclusion chromatography on Superose 6 were dissociated at pH 3.0, adjusted by the addition of 0.1 M glycine-HCl buffer (5). Solid phase-immobilized



**Figure 1** Possible structures of O-glycans in the hinge region of human IgA1.

**Table 1**

Binding of IgG from normal human sera to IgA1 and IgA2 myeloma proteins and Fab IgA1 with intact and modified hinge region glycans

Myeloma	IgG binding (OD 405 nm, 30 minutes)	n	Significance
IgA1 (Lat)	0.826 ± 0.615	10	
IgA1 (Pet)	0.660 ± 0.439	10	
IgA1 (Kni)	0.477 ± 0.271	10	
IgA1 (Mce)	0.419 ± 0.250	10	
IgA1 (Wil)	0.404 ± 0.420	10	
IgA1 (Ber)	0.388 ± 0.227	10	
IgA1 (Git)	0.218 ± 0.118	18	
IgA2 (Fel)	0.024 ± 0.012	10	$P < 0.0001^A$
Fab IgA1 (Ber)	0.116 ± 0.188	53	
De-O-glycosylated Fab IgA1 (Ber)	0.025 ± 0.036	53	$P < 0.0008^B$
Fab IgA1 (Ste)	1.989 ± 1.072	20	
De-O-glycosylated Fab IgA1 (Ste)	0.527 ± 0.560	20	

Data shown are mean ± SD. <sup>A</sup>IgA2 (Fel) vs. any of the IgA1 protein above; <sup>B</sup>vs. Fab IgA1 (Ber); <sup>C</sup>vs. Fab IgA1 (Ste).

inhibitors were added to dissociated CICs, and the suspension was neutralized with sodium bicarbonate to allow re-formation of CICs. A naturally undergalactosylated IgA1 myeloma protein (Mce) and an asialo-β1,3-agalacto-(a,a-) IgA1 myeloma protein (Mce) were immobilized on Sepharose 4B. The same solid phase was used to immobilize asialo-ovine submaxillary mucin (a-OSM), kindly provided by G. Hart (The Johns Hopkins University, Baltimore, Maryland, USA) and T. Gerken (Case Western Reserve University, Cleveland, Ohio, USA). *N*-acetylglucosamine (GlcNAc) and GalNAc immobilized on agarose were purchased from Sigma Chemical Co. Immobilized human serum albumin (HSA) served as negative control. After 2 hours' incubation at room temperature, the inhibitors and bound proteins were removed by centrifugation. An aliquot of the supernatant from each sample was then placed into wells of a microtiter plate coated with staphylococcal protein G (Sigma Chemical Co.). The captured IgG was detected by immunoassay using biotinylated F(ab')<sub>2</sub> fragment of goat anti-human IgG antibody. The affinity of IgG toward a particular inhibitor was estimated from the amount of IgG bound to protein G from a sample incubated with and without immobilized inhibitor.

**Subclass specificity of IgG with anti-IgA1 hinge region activity.** Sera (diluted 1:10) from IgAN patients and healthy controls were incubated in wells of a microtiter plate coated with the a,a-IgA1 proteins; identically treated IgA2 myeloma protein served as a control. The subclasses of captured IgG were determined with the following biotinylated mouse mAb's: clone JDC-1 (mouse IgG2a, IgG1-specific; Sigma Chemical Co.); clone HP6014 (mouse IgG1, IgG2-specific; Sigma Chemical Co.); clone HP6050 (mouse IgG1, IgG3-specific; Southern Biotechnology Associates Inc.); and clone HP6023 (mouse IgG1, IgG4-specific; Southern Biotechnology Associates Inc.).

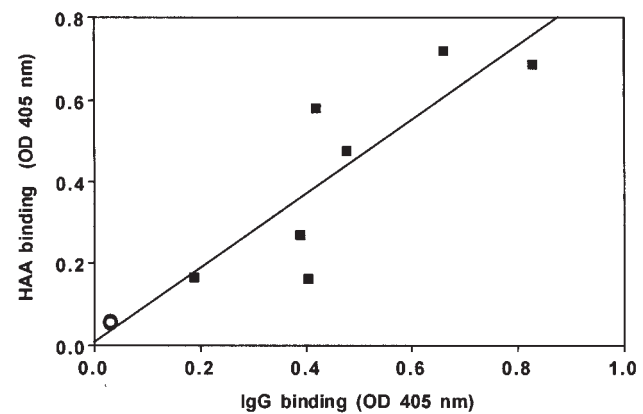
**Detection of IgA1 and IgM antibodies with anti-IgA1 hinge region glycan activity.** To determine whether immunoglobulins of isotypes other than IgG were bound to a,a-IgA1, sera (diluted 1:10) of IgAN patients and healthy controls were incubated in wells of a microtiter plate coated with the Fab fragment of IgA1 (Ste) protein that was shown to bind IgG. Biotinylated antibodies against human IgA1 (specific for the Fcα region, clone H69-7.1; ref. 29) and human IgM [F(ab')<sub>2</sub> fragment of goat anti-human IgM, heavy chain specific; BioSource International, Camarillo, California, USA] were used in the ELISA.

**Carbohydrate analyses.** Carbohydrate analyses were performed by gas chromatography. The gas chromatograph (model 5890; Hewlett-Packard, Sacramento California, USA) was equipped with a 25-m fused silica OV-1701 WCOT column (Chrompack, Bridgewater, New Jersey, USA), electron capture detector, and Hewlett-Packard model 3396 integrator (30). The monosaccharides were determined as trifluoroacetates of methyl-glycosides.

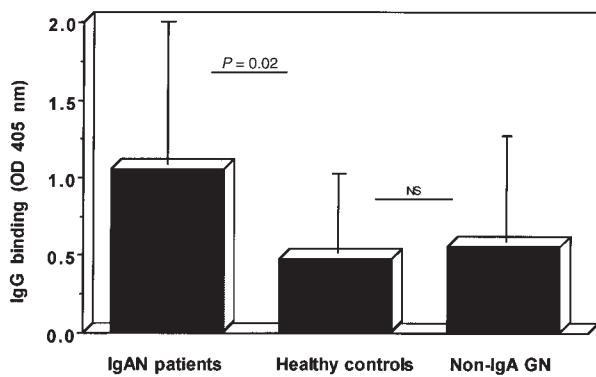
**Statistical analyses.** Comparisons between group means were performed using Student's *t* test. *P* values less than 0.05 were considered statistically significant.

## Results

**Interactions of human serum IgG with hinge region glycans of IgA1 myeloma proteins.** The binding of IgG from sera of normal individuals to various IgA1 myeloma proteins differed considerably, indicating structural heterogeneity of IgA1 proteins; binding to IgA2 protein was significantly lower (Table 1). IgG bound also to Fab fragments prepared from IgA1 myeloma proteins by incubation with IgA1 protease from *H. influenzae*, which cleaves IgA1 in the hinge region so that the *O*-linked glycans remain associated with the Fab fragment (24). Gas chromatographic carbohydrate analyses of Fab fragments from IgA1 Ber and Ste showed the predominant pres-

**Figure 2**

Correlation of binding of HAA and normal serum IgG to IgA myeloma proteins. The binding of serum IgG to IgA1 (filled squares) and IgA2 (open circle) myeloma proteins. The binding of IgG to IgA1 significantly correlated with HAA binding ( $r = 0.875$ ,  $P = 0.044$ ), indicating requirement of terminal GalNAc residues for IgG binding.



**Figure 3**  
The binding of serum IgG to Fab fragment of IgA1 (Ste) myeloma protein. Wells of microtiter plates were coated with Fab fragment of IgA1, incubated with diluted sera from 20 IgAN patients, 20 healthy controls, and 20 patients with non-IgA GN and subsequently with biotinylated mAb specific for IgG, avidin-alkaline phosphatase, and phosphatase substrate. Data shown are OD at 405 nm, mean and SD. Statistical significance is noted; NS, not significant.

ence of constituents of *O*-linked glycans (GalNac, Gal, and NeuNac), whereas the typical constituents of *N*-linked sugars, mannose, and GlcNac were present in trace amounts, and fucose was not detected. These preparations varied considerably in their ability to bind serum IgG, owing to differences in the galactosylation or sialylation of the hinge region glycans. Total or partial cleavage of glycans from Fab by incubation with neuraminidase and endo-*O*-glycosidase resulted in a significant loss of the ability to bind IgG ( $P = 0.0008$  and  $0.0001$ , respectively).

These data indicated that the binding site for IgG was in the area of the hinge region glycans. The hinge region glycans of normal serum IgA1 consist mostly of mono-, di-, tri-, and tetrasaccharides linked to serine or threonine (16, 18–20) (Figure 1). The IgG binding to IgA1 myeloma proteins correlated ( $r = 0.875$ ,  $P = 0.044$ ) with the binding of HAA, a lectin specific for GalNac (Figure 2). The involvement of GalNac as one of the antigenic determinants for IgG with anti-*a*,*a*-IgA1 binding activity was also suggested by experiments in which the binding of IgG to *a*,*a*-IgA1 was partially inhibited by HAA. In conclusion, the results suggested that IgG antibody with specificity to serine- or threonine-linked GalNac residues is present in sera of IgAN patients and healthy individuals.

The increased binding of HAA to IgA1 in IgAN patients, and the correlation between IgA1 binding of HAA and serum IgG, led us to investigate whether sera of IgAN patients contain higher levels of IgG with specificity toward hinge region glycans. A significantly larger amount of IgG was bound to microtiter plates coated with IgA1 or Fab fragment of an IgA1 protein incubated with sera from IgAN patients when compared with those from healthy controls (Table 2). The binding of IgG from sera of patients and of controls

to IgA2 myeloma protein and to desialylated/de-*O*-glycosylated Fab fragment of IgA1 was negligible (Tables 1 and 2).

Sera from patients with IgAN and non-IgA GN, and from healthy controls, were tested for the presence of IgG antibodies reacting with Fab fragment of IgA1 (Ste) myeloma protein. Although IgG from IgAN patients exhibited elevated binding when compared with healthy controls ( $P = 0.02$ ), no significant difference was detected between controls and patients with non-IgA GN (Figure 3). Likewise, no statistically significant difference was detected between the binding of IgG from healthy controls and patients with non-IgA GN when *a*,*a*-IgA1 myeloma protein (Mce) was used instead of the Fab fragment.

To determine the molecular form of serum IgG that binds to *a*,*a*-IgA1, serum fractions obtained by size-exclusion chromatography on Superose 6 column were incubated with *a*,*a*-IgA1 immobilized in a microtiter plate and subsequently with biotinylated antibody specific for human IgG. IgA1 bound to uncomplexed IgG but not to IgG in CICs. This observation suggested that all binding sites of IgG in CICs were occupied. Serum IgG from a healthy individual with specificity to *a*,*a*-IgA1 was purified by affinity chromatography on immobilized *a*,*a*-IgA1. When tested by ELISA, this IgG antibody bound to *a*,*a*-IgA1 and to Fab fragment of IgA1 myeloma protein, but not to IgA2, which was used as a control. Therefore, we concluded that IgG antibodies to IgA1 with specificity to hinge region glycans were present in an uncomplexed form in normal sera and in elevated levels in sera of IgAN patients.

**Subclass specificity of IgG with anti-*a*,*a*-IgA1 binding activity.** The IgG subclass most frequently reacting with IgA1 with modified glycans was IgG2; IgG1 and IgG3 were less frequent, and the reaction with IgG4 was negligible (Figure 4). Within individual IgG subclasses, no significant difference was found between specimens from IgAN patients and healthy controls.

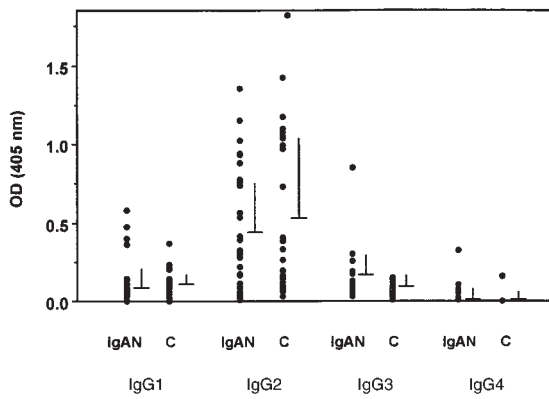
**Specificity of IgG antibodies in IgG-IgA1 CICs.** The antibody specificity of IgG in CICs was examined in fractions prepared from sera of IgAN patients and healthy controls by size-exclusion chromatography (Figure 5). Fractions containing HAA-binding protein were pooled

**Table 2**

Binding of serum of IgG from IgAN patients and healthy controls to intact and modified IgA myeloma proteins and their Fab fragments

Myeloma	IgG binding				Significance <sup>A</sup>
	(OD 405 nm, 30 minutes)				
	IgAN patients	<i>n</i>	Controls	<i>n</i>	
IgA1 (Ber)	0.429 ± 0.387	54	0.261 ± 0.198	54	$P = 0.0054$
Fab IgA1 (Ber)	0.202 ± 0.234	53	0.116 ± 0.188	53	$P = 0.0395$
De- <i>O</i> -glycosylated	0.037 ± 0.044	53	0.025 ± 0.036	53	NS
Fab IgA1 (Ber)					
Control (IgA2 Fel)	0.011 ± 0.009	20	0.024 ± 0.012	20	NS

Data shown are mean ± SD. <sup>A</sup>IgAN patients vs. controls. NS, not significant.



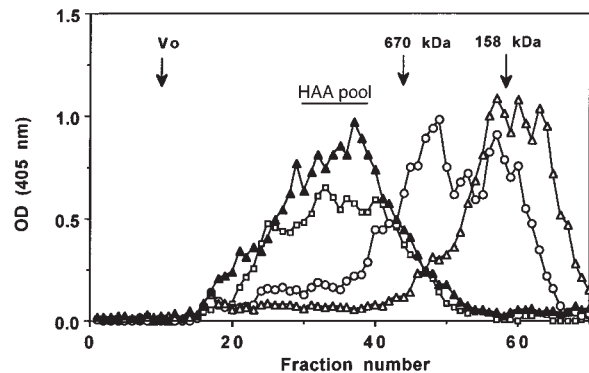
**Figure 4**  
Subclass specificity of serum IgG binding to a,a-IgA1. Wells of microtiter plates were coated with myeloma a,a-IgA1, incubated with sera (diluted 1:10) from 30 IgAN patients and 30 healthy controls (C) and subsequently with biotinylated mAb specific for IgG1, IgG2, IgG3, and IgG4; avidin-alkaline phosphatase; and phosphatase substrate. Data shown are OD at 405 nm, mean and SD.

and acidified to pH 3.0 to dissociate the immune complexes (5). Immobilized glycoprotein or monosaccharide inhibitors were incubated with aliquots of dissociated CICs, and the reaction was adjusted to neutral pH to allow re-formation of immune complexes. Percent inhibition of the complex formation was estimated from the concentration of IgG, determined by solid-phase immunoassay before and after the inhibition. Complex formation was inhibited by Sepharose-bound a,a-IgA1, intact myeloma IgA1, and a-OSM; inhibitory activity was also associated with agarose-bound GalNAc. The inhibitory activity of agarose-bound GlcNAc was close to that of immobilized HSA that was used as a negative control (Figure 6). The inhibitory activity associated with a-OSM and immobilized monosaccharide GalNAc indicated that IgG antibodies in high-molecular-mass CICs displayed specificity to Gal-deficient O-linked glycans of the IgA1 hinge region, as well as to structurally analogous glycans in other immunoglobulin-unrelated proteins.

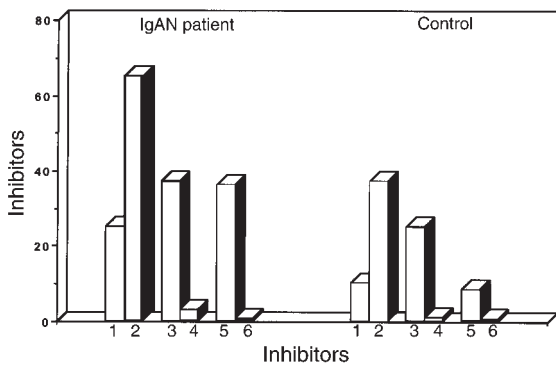
**Molecular forms of IgA1 in IgA1-IgG CICs from IgAN patients and healthy individuals.** To determine the presence of mIgA1 or pIgA1 in IgA1-IgG CICs, sera of an IgAN patient and a healthy control were subjected to HPLC in a size-exclusion column (TSK-5000) equilibrated with a buffer of neutral pH; fractions that exhibited the highest binding for a biotinylated lectin HAA were acidified to pH 3.0 to dissociate CICs and were subjected to a second HPLC separation on a size-exclusion column (TSK-3000) equilibrated in acidic buffer (pH 3.0). The elution profile was examined by ELISA, and the results indicated the presence of IgM, pIgA, mIgA, and IgG. The polymeric character of IgA in fractions containing IgA1-IgG CICs was confirmed by the presence of J chain, a typical component of polymeric IgA and IgM (25). In all serum specimens, the highest concentration of J chain was detected in a fraction that also contained the

maximal concentration of IgA reacting with HAA lectin (Figure 7). Because IgM was present in addition to pIgA, the concentration of J chain associated with IgA was determined before and after absorption of pooled fractions with solid phase-immobilized antibody to IgM. J chain was associated with IgA and IgM proteins and thus confirmed pIgA in these fractions.

**Investigations for the presence of IgA1 and IgM antibodies with anti-a,a-IgA1 binding activity.** To examine whether IgA1 and IgM antibodies with specificity toward the hinge region glycans are in the circulation, microtiter plates coated with the Fab fragment of the IgA1 myeloma protein (Ste) were incubated with sera from IgAN patients and healthy individuals and subsequently with biotinylated mAb specific for the Fc fragment (CH2 domain) of IgA1 (29), or antibodies against IgM or IgG. In IgAN patients, the binding of IgA1 and IgG to Fab fragment of IgA1 was higher (mean  $\pm$  SD:  $2.46 \pm 1.19$  and  $2.71 \pm 1.07$ , respectively) than in the control groups ( $1.85 \pm 1.30$  and  $1.99 \pm 1.07$ , respectively), although it was not statistically significant for IgA1. For IgG, this difference was statistically significant ( $P = 0.039$ ). The mean  $\pm$  SD for IgM binding to the Fab fragment was lower for patients ( $1.37 \pm 1.79$ ) than for the control group ( $1.99 \pm 1.07$ ; Table 3). Comparison of the control group with IgAN patients indicated that the difference in the binding of IgA1 and IgM was not statistically significant. Treatment with neuraminidase and O-glycosidase to remove O-linked sugars resulted in decreased average binding of IgG and IgA1 from both groups, whereas IgM binding did not change significantly. This observation indicated that IgA1 and IgG can recognize epitopes that require the O-linked sugars, whereas IgM may be specific for other epitopes. Analogous results were obtained for IgM when a,a-IgA1 myeloma protein, instead of Fab, was used for coating of microtiter plates.



**Figure 5**  
Distribution of IgM, IgA, IgG, and reactivity with HAA in serum fractions from Superose 6 column. Serum from an IgAN patient was fractionated on a Superose 6 column ( $0.9 \times 58.5$  cm); 0.25-mL fractions were collected and analyzed by ELISA for the content of IgM (squares), IgA (circles), IgG (open triangles), and reactivity with HAA (filled triangles). Fractions containing aberrantly glycosylated IgA were pooled (HAA pool) and used in the inhibition experiment.



**Figure 6**  
Inhibition of IgA1-IgG complex formation. IgA1-IgG CICs from an IgAN patient (left columns 1–6) and a healthy control (right columns 1–6) prepared by size-exclusion chromatography on Superose 6 were dissociated at pH 3.0. Aliquots of dissociated CICs were incubated with agarose- or Sepharose-immobilized inhibitors: 1, IgA1(Mce); 2, a,a-IgA1(Mce); 3, GalNAc; 4, GlcNAc; 5, a-OSM; and 6, HSA. The mixture was adjusted to neutral pH to allow re-formation of immune complexes, and the affinity of IgG toward the particular inhibitor was determined as described in Methods.

### Discussion

The presence of IgA-IgG CICs in most IgAN patients has been reported in many studies (for reviews, see refs. 2, 3, 5, 7, 31, 32). However, a search for potential exogenous or endogenous antigen as a component of such CICs has not been fruitful (4). The current study indicates that these CICs are composed of IgA1 with aberrantly glycosylated O-linked glycans in the hinge region and IgG, some of which had antibody activity directed at such antigenic determinants.

Several recent studies have shown that a portion of IgA1 molecules in sera of IgAN patients display alterations in O-linked (9–17), and perhaps N-linked (10), glycans. The most frequently encountered changes were restricted to Gal (9–17) and NeuNAc (14) in O-linked IgA1 hinge region glycans, as determined by reactivities with NeuNAc-, Gal-, and GalNAc-specific lectins and direct carbohydrate analyses. Studies of lectin reactivities of serum fractions from IgAN patients and from healthy individuals obtained by size-exclusion chromatography revealed Gal-deficient IgA1 molecules in high-molecular-mass fractions containing IgA1 and IgG (9). Because such IgA1-IgG CICs are dissociable at acid pH, we considered that the component immunoglobulin may involve antigen-antibody interactions with Gal-deficient IgA1 as an antigen and IgG as an antibody to altered IgA1. The rationale for such an interaction was based on several studies of diseases in which IgG and IgM antibodies to carbohydrates play

an essential role (33–38). In the rare disease mixed field polyagglutinability, also called Tn syndrome (34–36), blood elements including lymphocytes of T and B cell lineage, platelets, and/or erythrocytes are deficient in  $\beta$ 1,3-linked Gal in O-linked cell surface glycoproteins, resulting in the exposure of terminal GalNAc (33–37). Naturally occurring antibodies with specificity to terminal GalNAc interact with these cells causing their agglutination leading to tissue damage (38).

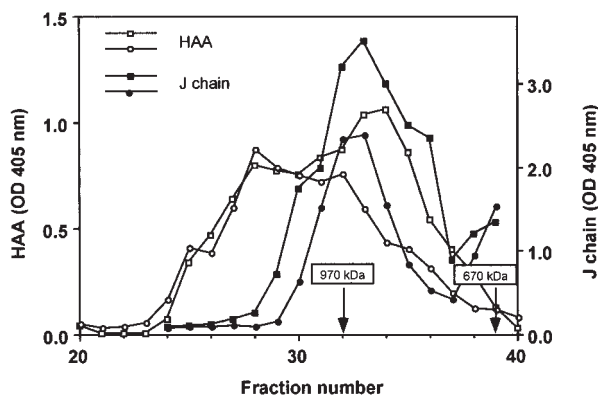
A number of microorganisms, including viruses (e.g., respiratory syncytial, Epstein-Barr, and herpes viruses; refs. 39–41) and bacteria (certain strains of streptococci; ref. 42), express GalNAc on their surface structures and are likely to induce corresponding IgG, IgM, and IgA antibodies. This is certainly the case with iso-hemagglutinins (anti-blood group A and B substances) induced in individuals with blood groups B, A, and O by bacteria (*Streptococcus pneumoniae* and *Escherichia coli*) that colonize mucosal surfaces (43). Such anti-A and anti-B antibodies recognize terminal carbohydrates in certain glycosidic bonds on surfaces of erythrocytes. By analogy, we postulate that naturally occurring IgG antibodies in the circulation of IgAN patients and, to a lesser extent, in normal individuals recognize terminal GalNAc in Gal-deficient hinge region O-linked glycans of IgA1. The increased levels of anti-GalNAc antibodies in IgAN patients probably result from preceding infections with microorganisms expressing GalNAc-associated epitopes; it is also possible that in IgAN, Gal-deficient IgA1 may further boost anti-GalNAc antibodies. The GalNAc specificity of IgG antibodies was shown by (a) increased binding of serum IgG to IgA1 that was modified by desialylation and removal of Gal residues from the hinge region glycans with exoglycosidases, and (b) decreased binding after removal of hinge region glycans by endo-O-glycosidase. Furthermore, competitive immunoassay of IgG from acid-dissociated IgA1-IgG CICs showed that IgA1 in these complexes can be partially displaced with (a) naturally or enzymatically undersialylated and undergalactosylated myeloma IgA1 (NeuNAc did not appear to be a significant part of the GalNAc epitope, as native or desialylated IgA1 was similarly effective in these experiments); (b) glycoproteins with glycan structure similar to that of hinge region gly-

**Table 3**

Binding of serum IgA1, IgM, and IgG from IgAN patients and healthy controls to Fab fragment of IgA1 (Ste) and partially de-O-glycosylated Fab fragment of IgA1 (Ste)

Serum source	Immunoglobulin	Immunoglobulin binding (OD 405 nm; 30 minutes) to			
		Fab IgA1 (Ste)	Significance <sup>A</sup>	De-O-glycosylated Fab IgA1 (Ste)	Significance <sup>A</sup>
IgAN	IgA1	2.46 ± 1.19	NS	0.99 ± 0.64	P = 0.038
Control	IgA1	1.85 ± 1.30		0.60 ± 0.50	
IgAN	IgM	1.37 ± 0.80	NS	1.71 ± 0.95	NS
Control	IgM	1.63 ± 0.89		1.54 ± 0.58	
IgAN	IgG	2.71 ± 1.07	P = 0.039	0.88 ± 0.64	NS
Control	IgG	1.99 ± 1.07		0.53 ± 0.56	

Data shown are mean ± SD. <sup>A</sup>IgAN patients vs. healthy controls for the same Ig isotype; n = 20. NS, not significant.



**Figure 7**

Distribution of HAA-reactive protein and J chain in serum fractions. Sera from an IgAN patient and a healthy control were fractionated on HPLC size-exclusion column TSK-5000. Aliquots of 0.25-mL fractions from the IgAN patient (open squares) and the control (open circles) were assayed for terminal GalNAc with biotinylated HAA. Aliquots of the same fractions from the IgAN patient (filled squares) and the control (filled circles) were assayed for J chain.

cans with free terminal GalNAc residues, such as a-OSM; or (c) agarose-bound GalNAc residues. Although evidence for deposition of IgA1-IgG CICs in the mesangium is circumstantial, recent studies suggest that IgG in the deposits is a risk factor in early IgAN (44). However, mesangial IgG deposits are not found in all cases of IgAN (7). The binding of serum IgA1 to Fab fragment of IgA1 with hinge region glycans containing free terminal GalNAc residues indicated that, in addition to IgG, naturally occurring IgA1 antibodies with specificity to hinge region glycans are also in sera of IgAN patients and healthy individuals. It was suggested that a nonimmunological mechanism might be responsible for formation of macromolecular IgA1 by self-aggregation of aberrantly glycosylated IgA1 molecules in IgAN patients (15). However, our data suggest that IgA1 is an antigen that binds to naturally occurring serum IgG and IgA1 in IgAN patients and healthy controls. We cannot rule out, however, that aggregation of aberrantly glycosylated IgA1 may play a role in the deposition to mesangium in vivo. Finally, glycan-modified IgA1 interacts with C3 (45) and mannose-binding lectin (46) that may increase the molecular mass of IgA1-containing CICs, resulting in inefficient clearance from the circulation and deposition in the mesangium.

In contrast to IgG and IgA1, we did not find naturally occurring IgM antibodies directed against hinge region glycans. It is likely that IgM antibodies in CICs from IgAN patients (5), and from controls or in kidney biopsy specimens from some IgAN patients (4), have specificity to other hinge region epitopes. The participation of the protein backbone of the hinge region and the role of serine- or threonine-linked GalNAc residues in generation of the antigenic site are presently unknown.

The hinge region of human IgA1 molecules has a highly unusual structure among the major human and

animal immunoglobulins. Contrary to an earlier proposal that human IgA2 arose from IgA1 by deletion of the hinge region, recent studies clearly indicate that IgA2 is a phylogenetically older form and that IgA1 was generated by insertion of DNA encoding the hinge region (for review, see ref. 47). The IgA1 hinge region displays sequence homology to mucins, including the presence of O-linked glycans (48). The hinge region-linked glycans, NeuNAc and Gal in particular, are likely to mask the potential antigenic sites in fully glycosylated IgA1 molecules. However, the absence of NeuNAc and Gal residues, due to cleavage with glycosidases or deficiency of specific glycosyltransferases, exposes GalNAc and possibly protein backbone-associated antigenic determinants on the hinge region. These are recognized by corresponding naturally occurring antibodies in the circulation. The importance of GalNAc as the dominant epitope is strengthened by the fact that (a) removal of GalNAc from Fab fragments of IgA1 decreases the reactivity with IgG (see Table 2), and (b) IgG antibodies to  $\alpha_1$ -IgA1 are predominantly in the IgG2 subclass, which is well known to be associated with anti-carbohydrate antibodies (49, 50).

What are the biological consequences of Gal deficiency and the formation of CICs? Circulating IgA1 has a relatively short half-life of 5–6 days (51). Approximately 90% of circulatory IgA is catabolized primarily in the liver by hepatocytes (52–55) that express the asialoglycoprotein receptor (ASGP-R) (52–54, 56–58). Before reaching the ASGP-R, molecules of glycoproteins must exit the circulation to enter the space of Disse through fenestrae found in the vascular endothelial cells. These fenestrae have effective pore sizes of 180–250 Å (59); larger molecules, such as IgM and its complexes, remain in the circulation and may be catabolized or deposited elsewhere (60). Consequently, large CICs do not penetrate this molecular sieve because of their size. Glycoproteins with terminal Gal or GalNAc that enter the space of Disse and interact with ASGP-R in the presence of calcium are readily internalized and degraded (61–63). Removal of Gal from the IgA1 hinge region does not reduce the hepatic clearance because the next proximal carbohydrate, GalNAc, is recognized by ASGP-R with even higher efficiency than the terminal Gal (63). Therefore, a mere Gal deficiency of O-linked glycan chains should not reduce the catabolism of IgA1. However, the presence of mainly IgG, but also IgA1 and possibly IgM, antibodies bound to GalNAc residues in the hinge region likely prevents the interaction of GalNAc with ASGP-R. In addition, an increased molecular mass of CICs comprising pIgA and IgG may hinder penetration through endothelial fenestrae, resulting in the diversion of such CICs from the catabolic pathway. Whether CICs that escape the liver-mediated clearance are identical to those in the mesangium is at present uncertain. Nevertheless, CICs and mesangial deposits share many common features, including the exclusive presence of IgA1 (partially in the polymeric form) (5, 64), C3, and IgG or IgM (2, 4).



A recent report suggests a possibility that the Gal deficiency of IgA1 may be due to a deficiency of  $\beta$ 1,3 galactosyltransferase in a fraction of IgA1-producing B cells (65). Further studies are required to determine whether the mesangial deposits contain IgA1 molecules deficient in  $\beta$ 1,3-linked Gal in O-linked glycans of the hinge region. Circulating IgG and IgA1 antibodies to  $\alpha$ , $\alpha$ -IgA1 hinge region GalNAc may explain why mesangial deposits in some patients contain IgG, in addition to IgA1, whereas other patients exhibit staining only for IgA1. Interestingly, serum antibodies to  $\alpha$ , $\alpha$ -IgA1 were predominantly in the IgG2 subclass and to lesser extents in the IgG1 and IgG3 subclasses. However, in other reports, the IgG in mesangial deposits were predominantly of the IgG1 and IgG3 subclasses (4, 66). This difference may be due to 2 factors. Human mesangial cells express Fc $\gamma$ RIII receptor (67) that binds IgG1 and IgG3 but only minimal amounts of IgG2. Thus, the mesangial cells may selectively bind IgA-CICs with IgG1 and IgG3. The IgG2-IgA complexes may be cleared by a different mechanism, possibly by binding to the Fc $\gamma$ RII receptors on other tissues. Second, monoclonal anti-IgG antibodies from several sources display variable specificities in different assays (68). Parallel studies of IgG subclass association of anti-GalNAc antibodies in serum, CICs, and mesangial deposits using identical mAb's are therefore needed.

The antigenic specificity of IgM, which is also frequently detected in mesangial deposits in IgAN (4), is not known. The relative proportion of such antibodies, as well as their affinity and, especially, avidity (higher in immunoglobulins with multiple binding sites), likely play important roles in the formation of CICs and their probable mesangial deposition.

### Acknowledgments

This work was supported by grant DK-49358 from the National Institutes of Health. The authors express their appreciation to Rhubell Brown and Rose Kulhavy for their technical assistance and to Catherine Barker for help with collecting clinical samples. Ovine submaxillary mucin was kindly provided by T. Gerken and J. Hart. Monoclonal antibody specific for IgA1 was kindly provided by J. Radl (TNO, Leiden, the Netherlands).

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