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Pathogenic role of (S)IgA in IgA nephropathy

Oortwijn, B.D.

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CHAPTER 5

Differential glycosylation of polymeric and monomeric IgA: a possible role in glomerular inflammation in IgA nephropathy

Beatrijs D. Oortwijn[1]*, Anja Roos[1]*, Louise Royle[2], Daniëlle J. van Gijlswijk-Janssen[1], Maria C. Faber-Krol[1], Jan-Willem Eijgenraam[1], Raymond A. Dwek[2], Mohamed R. Daha[1], Pauline M. Rudd[2], and Cees van Kooten[1]

** Both authors contributed equally to this study*

[1]Department of Nephrology, Leiden University Medical Center, Leiden, the Netherlands. [2]Glycobiology Institute, Department of Biochemistry, Oxford University, Oxford OX1 3QU, United Kingdom.

Summary

IgA nephropathy (IgAN) is characterized by mesangial deposition of polymeric IgA1 and complement. Complement activation via MBL and the lectin pathway is associated with disease progression. Furthermore, recent studies have indicated a possible role for secretory IgA. IgAN is associated with abnormalities in circulating IgA, including aberrant O-linked glycosylation. The present study characterized and compared functional properties and N-linked glycosylation of highly purified monomeric and polymeric IgA from IgAN patients and controls. Total serum IgA was affinity-purified from patients (n= 11) and controls (n= 11) followed by size-separation. Polymeric but not monomeric IgA contained secretory IgA, and its concentration was significantly higher in IgAN patients than in controls. Both in IgAN patients and in controls, IgA binding to the GalNAc-specific lectin HAA and to MBL was much stronger for polymeric than for monomeric IgA. Furthermore, binding of IgA to mesangial cells was largely restricted to polymeric IgA. Binding of polymeric IgA to mesangial cells resulted in increased production of IL-8, predominantly with IgA from IgAN patients. Quantitative analysis of N-linked glycosylation of IgA heavy chains showed significant differences in glycan composition between monomeric and polymeric IgA, including the presence of oligomannose exclusively on polymeric IgA. In conclusion, binding and activation of mesangial cells, as well as lectin pathway activation is a predominant characteristic of polymeric IgA as opposed to monomeric IgA. Furthermore, polymeric IgA has different N-glycans, which may recruit lectins of the inflammatory pathway. These results underscore the role of polymeric IgA in glomerular inflammation in IgAN.

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INTRODUCTION

Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis. The disease leads to progressive renal failure in a substantial proportion of patients. The hallmark of this disease is deposition of IgA in the glomerular mesangium, together with markers of complement activation (1,2). It is generally thought that this mesangial IgA mainly consists of IgA1 and is mostly polymeric (3). The composition of polymeric forms of IgA in serum is diverse and may include dimeric IgA, secretory IgA (SIgA), CD89 (FcαRI) /IgA complexes, IgA immune complexes and IgA-fibronectin complexes (4-6). Serum dimeric IgA consists of two IgA molecules linked with J chain, whereas SIgA in addition contains secretory component, derived from the mucosal epithelium.

Deposition of circulating IgA in the mesangium leads to renal inflammation, potentially involving direct interactions of IgA with resident and infiltrating cells in the glomerulus, as well as complement activation. The inflammatory process results in renal injury. Although the mechanism of IgA deposition in the renal mesangium of IgAN patients has been a subject of intensive research during the last decades, the pathogenesis of IgAN is still incompletely characterized. A number of studies provided evidence for a mesangial IgA receptor, which is involved in mesangial cell activation by IgA in vitro (7-9).

Glomerular IgA deposition is associated with activation of the complement system (5), involving the alternative pathway and the lectin pathway of complement (10). Recent studies indicate deposition of MBL, a major recognition molecule of the lectin pathway of complement, in a subpopulation of patients in association with a more severe renal injury (10,11), whereas in vitro studies demonstrated binding of MBL to polymeric serum IgA (12). Glomerular complement activation can enhance renal injury via the pro-inflammatory effects of the complement activation cascade.

Studies in IgAN patients who received a renal transplant showed recurrence of mesangial IgA deposition in a high number of cases (13). Vice versa, the accidental transplantation of a kidney with mesangial IgA deposition into a recipient without IgAN resulted in spontaneous disappearance of IgA deposits following transplantation (14). These studies strongly suggest that IgAN is a systemic disease rather than a disease of the kidney.

On basis of these data, abnormalities in IgA are hypothesized to be involved in the pathogenesis of IgAN. Therefore, circulating IgA from IgAN patients has been extensively studied. Serum from IgAN patients contains higher concentrations of IgA (15,16). Recently, our group showed low concentrations of circulating secretory IgA in IgAN patients and controls, whereas IgAN patients with a high serum concentration of SIgA showed more hematuria (17). Furthermore, SIgA accumulated in glomerular IgA deposits, suggesting a pathogenic role for SIgA in IgAN (17). Several studies also focused on IgA glycosylation, showing aberrant O-glycosylation on circulating IgA from IgAN patients, resulting in increased Tn antigen (GalNAcα1-Ser/Thr) residues (18,19). This undergalactosylated IgA1 may lead generation of circulating IgG-IgA1 complexes (20). O-linked glycans are present on IgA1 but not IgA2, whereas IgA1 and IgA2 heavy chains both contain several N-glycosylation sites (21). The galactosylation of the N-glycans is not different between IgAN

patients and controls (22). However, the complete structure of N-linked glycans on IgA has not been studied in IgAN.

Functional studies with purified IgA from IgAN patients suggested an increased interaction of IgA from IgAN patients with mesangial cells as compared to IgA from healthy individuals (23), although this is still controversial (24). Furthermore, after stimulation of mesangial cells with IgA from IgAN patients, the production of proinflammatory cytokines and chemokines was shown to be increased (25), possibly involving (26,27) undergalactosylation of IgAN IgA (28).

The aim of the present study was to characterize and compare the molecular composition and functional properties of monomeric and polymeric serum IgA from IgAN patients and controls. Therefore, we analysed highly purified total serum IgA from patients and controls in a number of aspects that are potentially important in the pathogenesis of IgA nephropathy, including interaction with lectins and mesangial cells. The results show clear functional differences between naturally occurring polymeric and monomeric serum IgA both for patients and controls. The most obvious difference that was noted between IgA isolated from IgAN patients and from controls was an increased fraction of SIgA in polymeric IgA from IgAN patients. Furthermore, we demonstrate that polymeric IgA differs from monomeric IgA in its composition of N-linked glycans.

MATERIAL AND METHODS

Human subjects

In the present study, we obtained serum from 11 healthy volunteers and 11 patients with primary IgAN (Table 1). All patients had biopsy-proven IgA nephropathy. None of these patients had clinical or laboratory evidence of Henoch Schönlein purpura, systemic lupus erythematosus or liver disease, or received immunosuppressive therapy. A healthy control group was selected and matched for gender. The mean age of the controls was somewhat lower; however we had no indications that this affects the biochemical properties of IgA. Renal function was non stable in 5 of the 11 patients with serum creatinine ranging from 203 to 366 µmol/L. The study was approved by the ethical committee of the Leiden University Medical Center. All individuals gave informed consent.

Table 1 Clinical characteristics of the patients with IgA nephropathy and control persons

	IgAN patients	controls
Number	11	11
Male/ female	8/3	9/2
Mean age (*years)*	48	33
Mean serum creatinine (*µmol/L;* range)	190 (82-366)	ND
Proteinuria (0 to 3+; median)	1+	ND
Erythrocyturia (0 to 5+; median)	1+	ND

IgA purification

IgA was precipitated from serum using $(NH4)_2SO_4$ at 50 % saturation, followed by affinity chromatography using HisA43 (mAb against human IgA, kindly provided by dr J. van den Born, Free University Medical Center, Amsterdam) coupled to CNBR-activated Sepharose 4FF (Amersham). A column of 15 ml was loaded with gamma globulin precipitate corresponding to 10 ml serum, using 0.5 x PBS as a running buffer, followed by washing with 90 ml 0.5 x PBS. Fractions of 3 ml were collected. To remove non-specifically bound proteins, the column was washed with 70 ml 1 M NaCl. Finally, bound IgA was eluted with 100 ml 0.1 M glycine/ 0.3 M NaCl (pH 2.8). Fractions were neutralized with 1 M Tris (pH 8.0). Fractions that contained IgA, as assessed by ELISA (29), were pooled, dialysed against PBS containing 2 mM EDTA, and applied to a mixture of protein G/ anti-human IgM (HB57)-Biogel A5 to remove residual contaminating IgG and IgM, followed by concentration and size-separation with a HiLoadTM 16/60 HR200 Superdex prep grade gel filtration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands), run in 50 mM NH_4HCO_3. Fractions were assessed for the presence of IgA and total protein. On basis of the protein profile, IgA-containing fractions were pooled into polymeric IgA (eluted at 44-50 ml) and monomeric IgA (eluted at 50-60 ml). These pools were analysed for total IgA, IgA1 and IgA2 content using ELISA (29). The percentage of polymeric IgA was quantified using calculation of the area under the curve on basis of the gel filtration profile.

MBL binding ELISA

MBL was purified from pooled plasma obtained from healthy human donors, as described before (12), resulting in a preparation of MBL in complex with its associated serine proteases (MASPs). MBL binding was studied by ELISA, in which 5 µg/ml IgA, or human serum albumin (HSA) as a control, was coated , followed by blocking with PBS/BSA, incubation with MBL (2 µg/ml) and detection of MBL binding as described (12). For inhibition experiments, MBL was pre-incubated with MgEGTA (10 mM), D-mannose or L-mannose (100 mM, from Sigma).

Activation of C4 via the lectin pathway

Activation of C4 by MBL-MASP complexes was measured as described before (12). In brief, incubation of MBL-MASP-complexes on coated IgA was followed by incubation with purified C4 and detection of C4 binding.

HAA binding

IgA was assessed for binding to biotinylated *Helix Aspersa* (HAA, Sigma) lectin, known to recognize terminal GalNAc. NUNC Maxisorp plates were coated with 5 µg/ml IgA or HSA as a control, in carbonate buffer (pH 9.6), overnight at room temperature. After washing with PBS/ Tween and blocking for one hour with PBS/ 1 % BSA, wells were incubated with 5 µg/ml biotinylated HAA in PBS/ 1 % BSA/ 0.05 % Tween. Binding of HAA was detected with HRP-conjugated streptavidin (Zymed). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. at 415 nm was measured.

SIgA ELISA

In order to quantify SIgA levels in isolated IgA, a sandwich ELISA specific for SIgA was used as described before (17). Briefly, plates were coated with a mAb against secretory com-

ponent (NI194-4), followed by incubation with IgA and detection of IgA binding.

Glycosidase treatment of IgA

Detection of undergalactosylated IgA with lectins could be hampered by the presence of sialic acids. To get a clear and full picture of the galactosylation, we treated IgA with neuraminidase and checked for binding to HAA and MBL. IgA (5 µg/ml) and HSA were coated, followed by blocking, and subsequent incubation with 100 mM sodium acetate pH 5.0, with or without 10 mU/ml Neuraminidase from Arthrobacter ureafaciens (Roche, Mannheim, Germany), for 3 hours at 37 ºC. Subsequently HAA and MBL binding were assessed as described above.

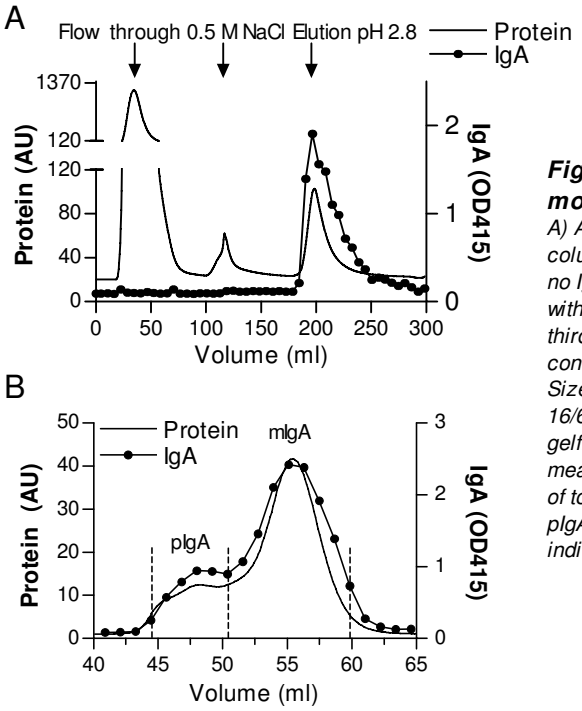

Figure 1. Purification of monomeric and polymeric IgA.
A) Affinity purification of IgA with an anti-IgA column. First peak is flow through (protein, no IgA), the second peak is after washing with 0.5 M NaCl (protein, no IgA), and the third peak is after acid elution. This peak contains IgA as detected with ELISA. B) Size-fractionation of IgA on a HiLoadTM 16/60 HR 200 Superdex prep grade gelfiltration column. All fractions were measured for total protein and the presence of total IgA by ELISA. IgA was pooled in pIgA (44-50 ml) and mIgA (50-60 ml) as indicated.

Cell culture

Normal human mesangial cells (NHMC, Cambrex, USA) were expanded according to the protocol provided by the manufacturer in mesangial cell basal medium with supplements (Cambrex, USA). Experiments with NHMC were performed in RPMI with 10 % FCS, 1 % non-essential amino acids, 0.5 % transferrin/insulin/selenium, 1 % sodium pyruvate, and 1 % L-glutamine (all purchased at Gibco/ life Technologies, Paisley, Scotland). AMC11, a spontaneously growing mesangial cell line of adult human origin (kindly provided by Prof. Holthofer, Helsinki), was cultured in DMEM with 10 % FCS. Cells were harvested by trypsinization.

Flow cytometry

Cells were washed with FACS buffer (0.5 x PBS containing 1 % BSA/ 2.8 % glucose/ 0.01 % NaN_3) and incubated with monomeric and polymeric IgA. Following incubation for 1 hour at 4 º C, cells were washed and incubated for one hour at 4 º C with monoclonal anti-IgA mAb 4E8 (IgG1) (29). IgA binding was visualized using PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) and fluorescence intensity was assessed by flow cytometry (FACSCalibur, Cell quest software; BD Biosciences). Dead cells, identified by propidium iodide uptake, were excluded from analysis.

Cytokine analysis

Production of IL-8 and MCP-1 was measured in supernatants of cultured mesangial cells. Prior to stimulation, cells were transferred to 96-wells plates (Costar, Corning, NY) at a den-

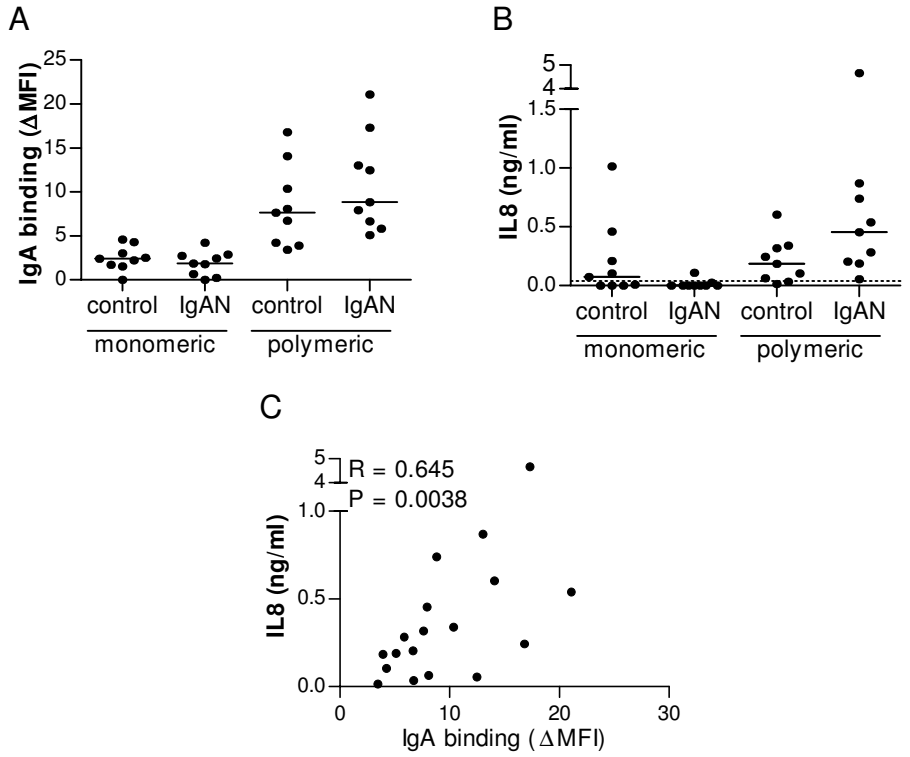

Figure 2. Increased binding and stimulation of mesangial cells with polymeric IgA. A) Normal human mesangial cells were incubated with different *molecular forms of IgA (200 µg/ml) from IgAN patients and controls, and assessed for IgA binding by flow cytometry. Depicted is the mean fluorescence intensity after subtracting the isotype control. B) Human mesangial cells (15 x 103 cells/well) were stimulated with different molecular forms of IgA (200 µg/ml). After 72 hours supernatants were harvested and tested for IL-8. Horizontal lines indicate the median, the dotted line represents the detection limit. IL-8 was undetectable in cultures without IgA. Polymeric IgA versus monomeric IgA (A, B): P < 0.01. C) Correlation between production of IL-8 after stimulation of mesangial cells with IgA and the binding of IgA to mesangial cells.*

sity of 15 x 10^3 cells per well and cultured overnight in culture medium with 0.5 % serum. Cells were cultured in the presence or absence of monomeric and polymeric IgA for 72 hours, in concentrations as indicated. The concentration of IL-8 and MCP-1 in culture supernatants was measured by ELISA as described (30,31).

N-glycan analysis

The IgA heavy chains were isolated on SDS-PAGE under reducing conditions and visualised by Coomassie staining. The N-Glycans were released from these excised gel bands by PNGase F, labelled with the fluorophore 2-aminobenzamide and analysed by normal phase (NP) HPLC with exoglycosidase sequencing as described (32).

Identification of Gel Bands by Mass Spectrometry.

The Coomassie-stained IgA heavy chain bands from an SDS-PAGE gel were excised and in-gel digested with trypsin (sequencing grade; Roche) as described (32).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test and the Wilcoxon signed rank test. The Spearman Rank correlation coefficient was used to analyze correlations. Differences in N-glycan composition were evaluated using the Student's T-test. Differences were considered statistically significant when p values were less than 0.05.

RESULTS

Polymeric IgA binds better to mesangial cells and induces more cytokine production after activation of mesangial cells

Human IgA was purified with an anti-IgA affinity column (Figure 1A). In the flow through no IgA was detectable whereas IgA was eluted by acid elution. The fractions containing IgA were pooled. Purified IgA was applied to a gel filtration column (Figure 1B), and fractions containing polymeric IgA (pIgA) and monomeric IgA (mIgA) were pooled as indicated.

To examine possible functional differences between monomeric and polymeric IgA from IgAN patients and controls, we investigated the binding of IgA to mesangial cells, as well as the cytokine response following stimulation. Polymeric IgA showed a 5.9-fold higher binding to mesangial cells than mIgA (P = 0.0003), but

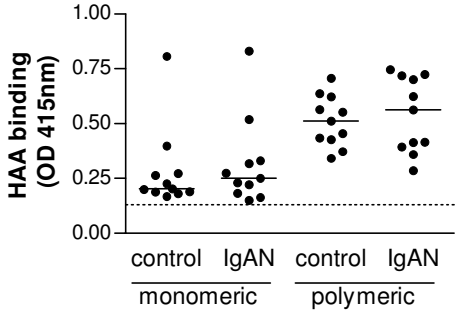

Figure 3. HAA binding to monomeric and polymeric IgA.
Monomeric IgA and polymeric IgA of IgAN patients and controls were coated in ELISA plates (5 µg/ml), followed by incubation with biotin-labeled HAA. Horizontal lines indicate the median, the dotted line represents binding to HSA. Polymeric IgA versus monomeric IgA: P = 0.0003.

there was no difference between IgAN patients and controls (Figure 2A).

Supernatants of the cells stimulated with IgA for 72 hours were tested for production of the chemokines IL-8 and MCP-1. The IL-8 production was significantly higher after stimulation of mesangial cells with polymeric IgA than after stimulation with mIgA (P = 0.010), whereas IL-8 production was undetectable in cultures without IgA (Figure 2B). Furthermore, IL-8 production tended to be higher upon stimulation with polymeric IgA from IgAN patients compared with that from controls (P = 0.077). A significant correlation was observed between binding of pIgA to mesangial cells and IL-8 production after co-culture (Figure 2C; R = 0.6450, P = 0.0038). Furthermore, stimulation with IgA also clearly enhanced production of MCP-1, and MCP-1 production correlated with IL-8 production after stimulation with different IgA samples (R = 0.59, P = 0.01). These functional data indicate intrinsic differences between monomeric and polymeric IgA.

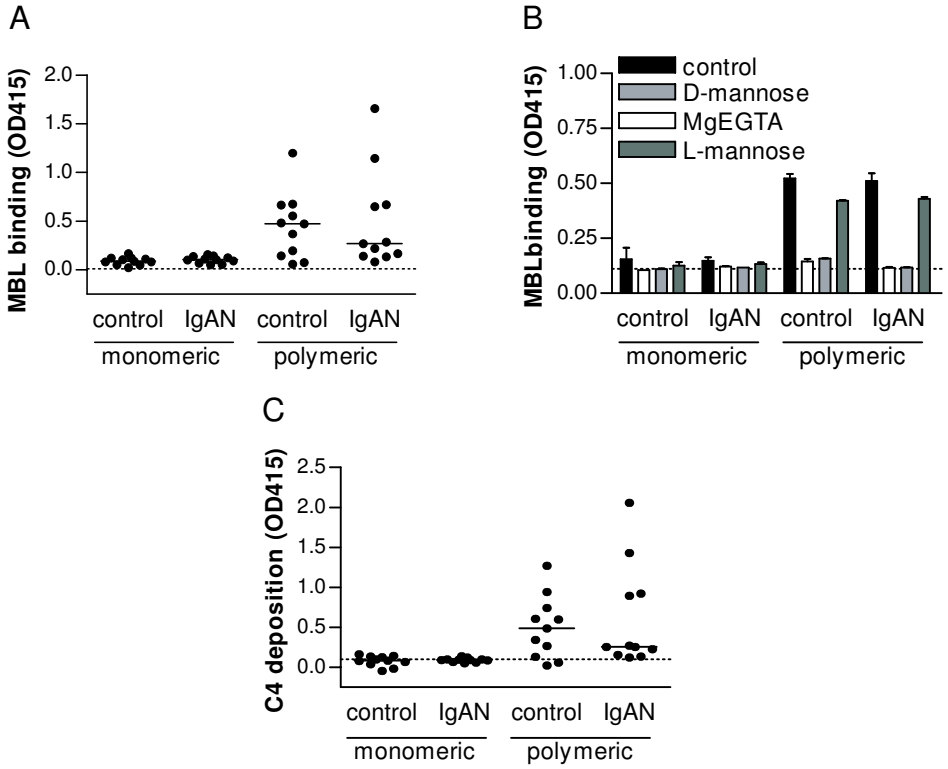

Figure 4. MBL binding to polymeric IgA from IgAN patients and controls.
Monomeric and polymeric IgA were coated in ELISA plates, followed by incubation with purified MBL-MASP complexes. A) Detection of MBL binding. B) MBL was pre-incubated with MgEGTA (10 mM), D-mannose (100 mM) or L-mannose (100 mM) before incubation on wells coated with IgA as indicated. MBL binding was detected. The horizontal dashed line represents the negative control C) Detection of C4 activation after incubation with purified C4. For each IgA sample, blanc values, obtained after incubation with C4 and/or detection antibodies without MBL, were subtracted. Dashed lines indicate OD values obtained with coating of HSA. Polymeric IgA versus monomeric IgA (A, C): P = 0.0001.

Interaction of polymeric IgA with HAA lectin

It has been reported that O-glycans of patients with IgAN contain more Tn antigen (GalNAc-Ser/Thr) compared to controls (18,33). Terminal GalNAc can be detected by specific lectins, including Helix Aspersa (HAA). We investigated the binding of HAA to IgA by ELISA (Figure 3). The binding of HAA to IgA from healthy individuals and IgAN patients was 4 fold higher for polymeric IgA than for monomeric IgA (P = 0.0003). However, we could not observe a difference in HAA binding between patients and controls.

MBL exclusively binds to polymeric IgA, resulting in C4 activation

We and others showed that IgAN is associated with complement activation via the lectin pathway (10,34). Therefore, the binding of MBL to purified IgA from patients with IgAN and controls was studied, showing that MBL binds to pIgA but not to mIgA with a high inter-individual variation, both for IgAN patients and controls (Figure 4A). Using parallel detection of immobilized IgA on plates, we confirmed that equal amounts of IgA were present in coated wells, indicating that differences in coating could not explain the observed differences (not shown). Binding of MBL to IgA was completely inhibitable by D-mannose and MgEGTA but not by L-mannose (Figure 4B), confirming that the C-type lectin domain of MBL was involved in binding to IgA.

Binding of MBL to pIgA from IgAN patients and from controls resulted in activation of purified C4 (Figure 4C), presumably involving C4 cleavage by MBL-associated MASP-2. This activation of C4 showed a strong correlation with MBL binding (R= 0.98, P < 0.0001 for pIgA).

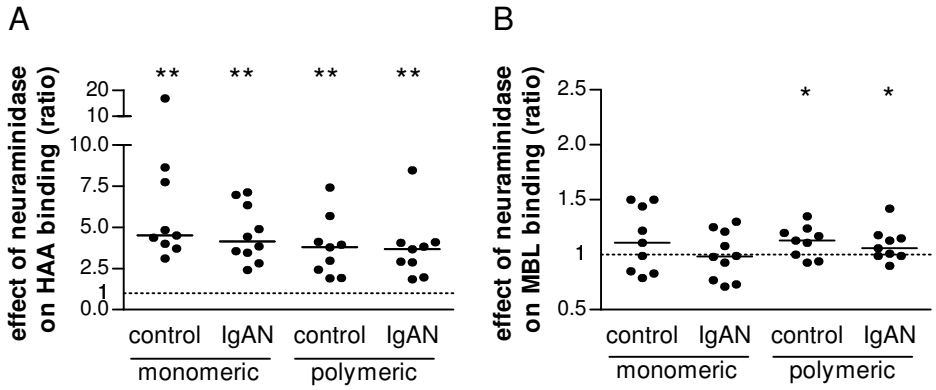

Figure 5: Increased interaction with HAA after treatment of IgA with neuraminidase.

*IgA (5 µg/ml) was coated in ELISA. After treatment with neuraminidase HAA binding (A) and MBL binding (B) were detected. Depicted is the ratio of non-treated and treated IgA after subtraction of background values, and the dotted line represents the non-treated IgA. * P < 0.05, ** P <0.01.*

Treatment of IgA with neuraminidase enhances its interaction with HAA

To examine whether sialic acids, commonly present on N-linked and O-linked glycans of IgA, might hamper the interaction of IgA with HAA and/or MBL we treated immobilized IgA with neuraminidase. After treatment of IgA the interaction with HAA increased significantly (3.8-fold for control mIgA, 3.1-fold for IgAN mIgA, 3.2-fold for control pIgA, 3.0-fold for IgAN pIgA; $P < 0.004$) (Figure 5A), suggesting the presence of sialylated Tn antigen, as the removal of the sialic acid exposes the GalNAc (Tn) epitope. In contrast, the binding of MBL to IgA was hardly affected by neuraminidase, only showing a minor increase after treatment of polymeric IgA (1.1-fold) (Figure 5B), consistent with the known specificity of MBL for glycans presenting 3,4 cis hydroxyls such as mannose to which sialic acids do not attach.

Molecular composition of monomeric and polymeric IgA from IgAN patients and controls

The results presented above indicate major functional differences between monomeric and polymeric IgA. We therefore investigated the molecular composition of monomeric and polymeric IgA from IgAN patients and controls. The size distribution of IgA from IgAN patients and controls was similar (mean % polymeric IgA, controls: 18.9 %, patients: 18.8%; $P = 0.89$). The IgA preparations were assessed for IgA1 and IgA2 content by ELISA. As described before (35), IgA2 is a minor constituent of human serum IgA. However, the relative amount of IgA2 was significantly higher in polymeric IgA (20 ± 4.1 %) as compared to monomeric IgA (9.2 ± 4.7 %) (Figure 6A) ($P< 0.0001$), suggesting that circulating IgA2 is more likely to be produced as polymeric complexes than circulating IgA1. The relative amount of IgA2 was similar in IgA from patients and controls.

Subsequently, we measured SIgA in monomeric and polymeric IgA from IgAN patients and controls. In agreement with the molecular size of SIgA, SIgA is exclu-

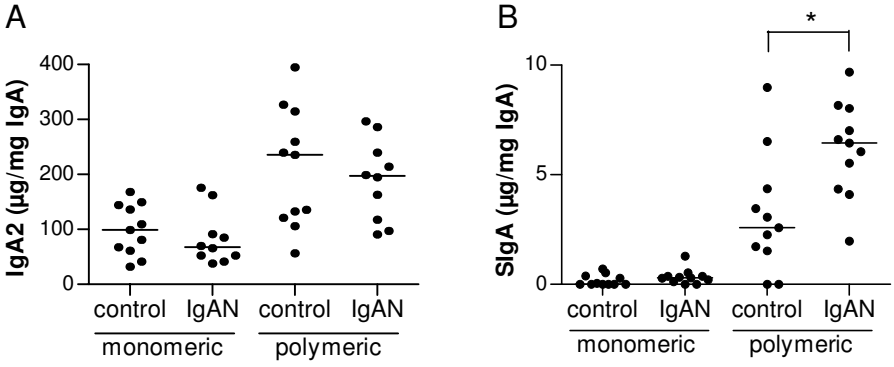

Figure 6. Increased concentration of SIgA and IgA2 in polymeric IgA.
*The relative content of IgA2 (A) and SIgA (B) in monomeric and polymeric IgA as assessed by ELISA. Horizontal lines indicate the median. Polymeric IgA versus monomeric IgA (A, B): $P < 0.0001$. SIgA content in pIgA of IgAN patients versus controls: * $P = 0.0152$.*

sively present in polymeric IgA (Figure 6B). SIgA comprised less than 1 % of total polymeric serum IgA. However, the proportion of SIgA present in polymeric IgA from IgAN patients is 2.5 times higher than in polymeric IgA from controls (P= 0.0152).

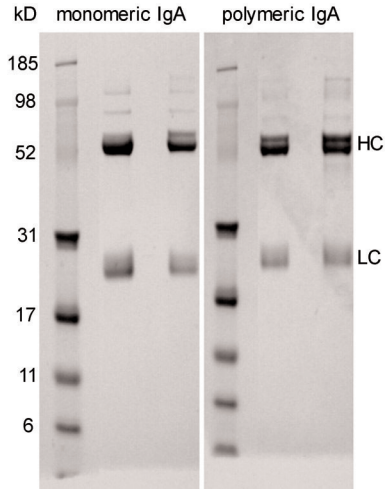

Figure 7. SDS-PAGE analysis of monomeric and polymeric IgA.
Separation of monomeric and polymeric IgA with 10% SDS-PAGE under reducing conditions shows heavy and light chains after staining with Coomassie. IgA heavy chains run as a doublet (upper and lower heavy chains).

Polymeric IgA shows a different composition of N-linked glycans compared to monomeric IgA

Recent studies showed that MBL is able to bind to N-linked glycans of IgG (36) and IgM (37). Based on the known structures of N-linked and O-linked glycans on IgA, it is more likely that MBL would bind to N-linked glycans than to the O-linked glycans. Furthermore, information on N-linked glycosylation of IgA in IgAN is not available. Therefore, we characterized the N-glycans of 6 monomeric and 6 polymeric IgA preparations in detail.

Heavy chains and light chains of monomeric and polymeric IgA were separated by SDS-PAGE (Figure 7). N-linked glycans were released via an in-gel digestion of the heavy chain and light chain bands of IgA using PNGase F. Isolated glycan samples were labeled with 2-aminobenzamide and run on normal phase HPLC. Consistent with earlier data (32), light chains of IgA were found not to be glycosylated (not shown). The elution pattern of heavy chains is shown in Figure 8A. The most prominent peaks, present between glucose units 8 and 10, represent complex glycans that are sialylated, as was demonstrated by a neuraminidase digestion (abs; Figure 8B). No obvious differences could be observed between N-glycans from patients and from controls. However, upon comparison of glycans from monomeric and polymeric IgA, a single peak at GU 6.2 was present in all polymeric IgA samples but absent in monomeric IgA. Digestion with mannosidase (jbm, Figure 8B) demonstrated that this is an oligomannose structure (Man 5), as schematically drawn in Figure 8A.

Using sequential enzyme digestions (Figure 8B), the glycan structures on these

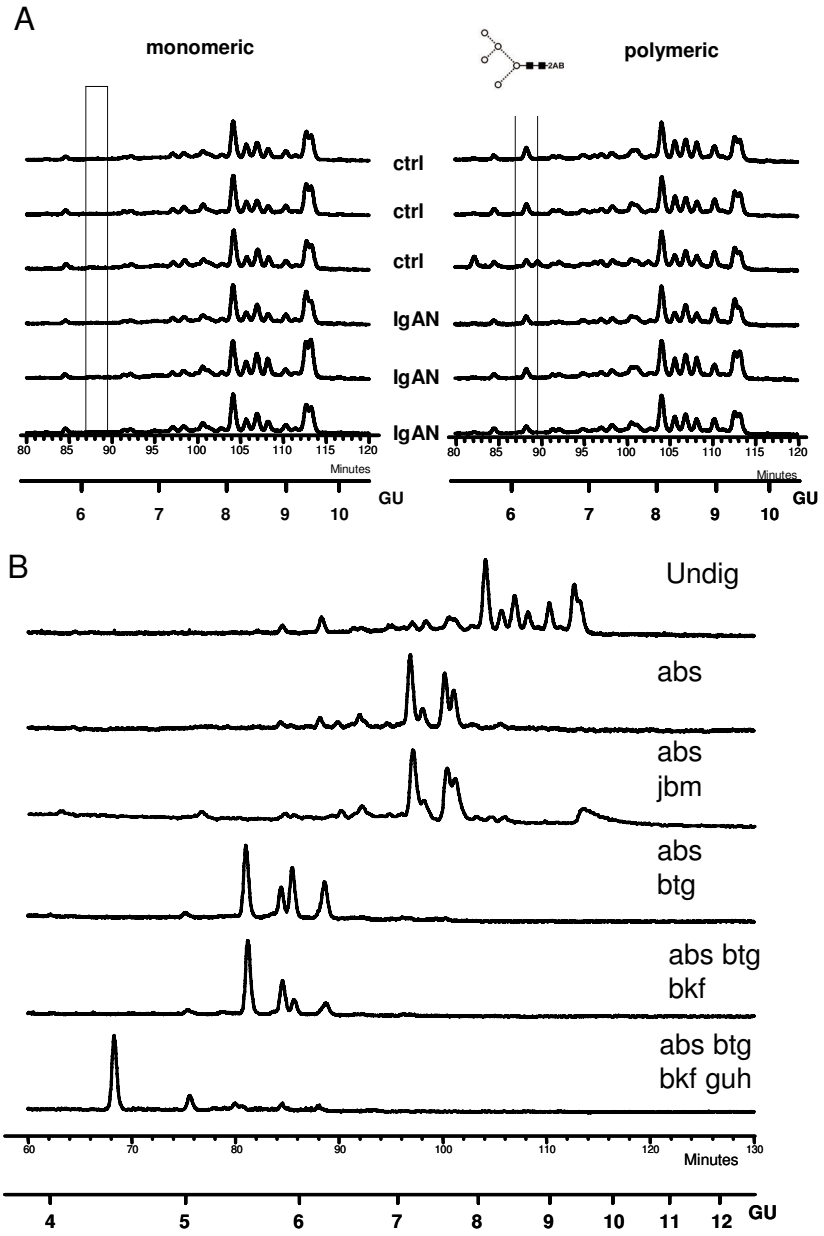

Figure 8. Analysis of N-linked glycans on IgA heavy chains.
A) Analysis of N-glycans of the heavy chain of polymeric IgA from controls and from patients with IgAN by NP-HPLC. The figures show the individual elution profiles following fluorescence detection. Retention times are standardized to a glucose oligomer ladder to give glucose units (GU). The boxed peaks are only present in polymeric IgA and are identified as oligomannose (Man 5), as indicated. B) Digestion of the N-glycans with A. ureafaciens sialidase (abs), jack bean α-mannosidase (jbm), bovine testes β-galactosidase (btg), bovine kidney α-fucosidase (bkf) and Streptococcus pneumoniae N-acetyl β-glucosaminidase (guh), followed by separation with NP-HPLC.

Table 2. Analysis of N-glycans of IgA heavy chains of monomeric and polymeric IgA

Major structure*	GU	monomeric							polymeric							
		Control			IgAN			Mean	Control			IgAN			Mean	P value
A2B	**5.80**	1.53	2.07	2.66	1.85	1.71	2.25	**2.01**	1.32	2.55	2.49	1.63	1.60	1.88	**1.91**	0.4469
Man 5	**6.17**	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	4.51	3.60	2.24	3.71	3.06	2.48	**3.27**	**0.0002**
A2G1[3] A2BG1[6]	**6.52**	0.66	1.02	0.84	1.03	1.21	1.67	**1.07**	0.95	1.72	1.39	1.11	1.14	1.24	**1.26**	0.3214
A2BG1[3] FA2G1[6]	**6.61**	1.48	1.68	1.89	1.21	1.67	2.28	**1.70**	1.23	1.09	1.88	1.06	1.16	1.66	**1.35**	**0.0187**
FA2G1[3] FA2BG1[6]	**6.92**	0.00	0.38	0.46	0.46	1.07	0.60	**0.50**	1.69	2.04	0.66	0.88	1.73	1.02	**1.34**	**0.0264**
FA2BG1[3] A2G2	**7.20**	2.10	1.89	2.23	1.87	1.37	2.08	**1.92**	2.16	2.65	2.75	1.32	2.09	1.09	**2.01**	0.7806
A2BG2	**7.37**	3.17	3.62	4.22	2.53	2.04	2.73	**3.05**	3.36	4.72	4.17	2.59	3.77	4.42	**3.84**	0.0663
FA2G2 A3G2	**7.65**	8.20	8.03	8.57	5.58	7.01	6.75	**7.36**	9.56	10.34	7.55	7.13	10.84	10.60	**9.33**	**0.0448**
FA2BG2	**7.73**															
A3BG2	**7.94**	2.51	1.52	2.28	1.66	1.61	1.12	**1.78**	2.00	2.32	3.64	1.69	2.74	2.96	**2.56**	0.0816
A2G2S1	**8.13**	20.22	21.15	20.65	21.64	16.24	20.75	**20.11**	17.43	17.28	19.15	21.81	15.89	17.83	**18.23**	**0.0338**
A3G3 A2BG2S1	**8.35**	7.81	7.20	5.56	5.66	6.65	6.29	**6.53**	8.40	8.64	6.50	6.44	7.64	8.04	**7.61**	**0.0017**
FA2G2S1 A3BG3	**8.50 8.52**	9.87	7.80	10.95	11.67	11.34	10.99	**10.44**	10.06	7.85	9.25	11.27	10.11	9.65	**9.70**	0.0709
FA2BG2S1	**8.72**	6.88	5.15	6.26	5.84	8.59	6.29	**6.50**	8.67	6.87	8.34	6.34	9.31	7.56	**7.85**	**0.0034**
A2G2S2	**9.03**	5.90	5.76	5.05	5.62	4.58	4.61	**5.25**	7.84	7.57	9.32	8.94	6.67	7.92	**8.04**	**0.0010**
A2BG2S2	**9.19**	2.80	2.28	2.06	3.03	2.51	2.55	**2.54**	1.96	2.14	1.79	1.93	1.67	2.68	**2.03**	**0.0493**
FA2G2S2	**9.40**	14.69	16.27	13.92	18.02	15.56	15.77	**15.70**	10.32	9.67	10.05	14.55	10.84	9.69	**10.85**	**0.0002**
FA2BG2S2	**9.48**	12.17	14.17	12.40	12.34	16.84	13.27	**13.53**	8.55	8.93	8.83	7.59	9.77	9.27	**8.82**	**0.0003**

N-glycans were identified on IgA heavy chains of monomeric and polymeric IgA from six individuals (3 IgAN patients and 3 controls, as indicated), using sequential exoglycosidase digestion as indicated in Fig. 8. For each structure, the percentage of total N-glycans is indicated. Differences between monomeric and polymeric IgA are evaluated by paired t-test. *Structural abbreviations: all N-glycans have two core GlcNAcs; F, core fucose linked α1-6 to inner GlcNAc; Man(x), number (x) of mannose on core GlcNAcs; A, number (x) of antenna (GlcNAc) on trimannosyl core; B, bisecting GlcNac linked β1-4 to inner mannose; Gx, number (x) of galactose on anntenne; Sx, number (x) of sialic acids on anntenne.

samples were identified and quantified (Table 2). Comparison of monomeric and polymeric IgA revealed that 3.3 % of the total glycan pool contained Man5 in polymeric IgA, whereas this structure is absent in monomeric IgA. Furthermore, the double sialylated glycans are under-represented in polymeric IgA as compared to monomeric IgA (29 % and 37 %, respectively, P = 0.001), resulting in a shift to smaller glycan structures on polymeric IgA. There were no significant differences between patients and controls. Together, the results indicate that N-linked glycosylation of the IgA heavy chain is significantly different between monomeric and polymeric IgA (Table 2).

DISCUSSION

Deposition of IgA in the renal mesangium is the primary characteristic of IgAN, responsible for glomerular inflammation and finally the development of renal failure. Based on earlier observations of mesangial IgA, this IgA is believed to be largely polymeric IgA1. In the present study we show that polymeric IgA, as opposed to monomeric IgA, from IgAN patients and from healthy controls shows increased binding to and activation of mesangial cells, and has a superior capacity to bind the complement-activating lectin MBL. These aspects of polymeric IgA are most likely to be involved in induction of glomerular deposition and inflammation. Furthermore, we provide evidence that polymeric IgA is differently glycosylated from monomeric IgA, as suggested by lectin binding studies and demonstrated by a direct identification of N-linked glycans. Noteworthy, the only obvious difference that was observed between IgA from IgAN patients and controls was a substantial increase in the fraction of SIgA in polymeric IgA from IgAN patients.

Comparisons between IgA isolated from patients and from healthy controls were reported previously (19,22,28,38-40). Most experiments were performed with either fractionated total serum without an IgA purification step (28,39,40) or with pooled serum IgA purified with Jacalin, a lectin that binds Gal 1-3GalNAc (39-41). In our study we purified IgA from individual patients and controls with an anti-IgA mAb. With this method we prepared monomeric and polymeric IgA that contains total and highly pure serum IgA without a preceding selection for certain IgA glycoforms. In contrast to methods using Jacalin, this method also enabled us to isolate the IgA2 present in serum.

Previous studies described that the binding of polymeric IgA to mesangial cells was higher than that of monomeric IgA (23,28) although this could not be reproduced by others (24). Moreover, the binding of patient IgA and that of in vitro degalactosylated IgA was higher than that of control IgA (24). In the present study, we confirm a prominent increase in mesangial cell binding of polymeric over monomeric IgA, but we did not detect a difference between patient and control IgA. Our studies further establish the pro-inflammatory properties of polymeric IgA, and demonstrate that IgA-induced chemokine production is correlates with the interaction of IgA with the mesangial cell surface. It is most likely that next to the increased binding of pIgA, also a more efficient receptor crosslinking will contribute to its proinflammatory action. In addition, also the observed biochemical properties of pIgA might contribute to this process. However, unlike previous investigations (27), this

property could not be specifically attributed to IgA derived from IgAN patients.

Next to the direct effects of IgA on mesangial cells, activation products of the complement system, involving both the alternative pathway and the lectin pathway, are likely to drive the local inflammatory process. Activation of the lectin pathway of complement via an interaction between MBL and IgA has been shown before (12), and in the present study we confirm and extend these data by showing that binding of MBL is a common feature of polymeric but not monomeric IgA isolated from different donors. Since ligand recognition by MBL requires multiply presented carbohydrates, MBL binding could be favored by the structure of polymeric IgA. Binding of MBL leads to activation of C4 presumably via activation of the C4-cleaving enzyme MASP-2. In a healthy situation, the binding of MBL to polymeric IgA could be involved in host defense. However, in IgAN, lectin pathway activation via polymeric IgA is unfavorable (10).

Many studies on IgA from IgAN patients focused on glycosylation. IgA is extensively glycosylated, both via N-linkages (IgA1 and IgA2) and O-linkages (IgA1) (21). It was consistently observed that serum IgA from IgAN patients contains smaller O-linked glycans, with less sialylation and galactosylation, than IgA from healthy controls (18). Previous investigations suggested that this was predominantly the case for monomeric IgA (40). Our experiments using HAA, a lectin that is commonly used to detect terminal GalNAc present on non-galactosylated O-linked glycans, suggested the presence of terminal GalNAc (Tn antigen) predominantly on polymeric IgA, both from patients and controls. This is in agreement with a previous study showing reactivity of HAA with high molecular weight serum proteins (28) and with data provided by Leung et al. (40). Binding of HAA was strongly increased by neuraminidase treatment, suggesting a high frequency of non-galactosylated O-linked glycans on IgA, that expose terminal GalNAc after enzymatic removal of sialic acid.

A detailed quantitative analysis of N-linked glycans on IgA heavy chains of monomeric and polymeric IgA revealed several significant differences between these molecular forms of IgA. In this respect, polymeric IgA consistently contained an oligomannose structure that was undetectable on all monomeric IgA preparations, and showed significantly less glycans with two terminal sialic acid residues. Recent studies showed that MBL can bind to certain glycoforms of human IgM, involving GlcNAc-terminated glycans and oligomannose structures on the IgM heavy chains (42). Therefore, the presence of specific glycans on polymeric but not monomeric IgA might also be involved in its recognition by MBL. At present it is unknown whether a specific glycosylation pattern of the heavy chain of polymeric IgA is involved in the polymerization of IgA, and/or whether this merely is related to the conditions present during production of the different forms of IgA. Earlier studies from our group indicated that polymeric serum IgA contains dimeric IgA linked with J chain, as well as complexes of monomeric IgA linked via other mechanisms (43). In the first case, polymerization takes place in the B cell, and the presence of oligomannose, which is a premature glycan structure, might suggest the ER as a possible location for polymerization, which prevents further synthesis of the glycan structure by steric hindrance. In the latter case, polymerization might take place outside the B cell.

Part of the observed differences in glycosylation between monomeric and poly-

meric IgA might also be explained by the presence of SIgA in the polymeric fraction of serum IgA, since the SIgA heavy chain N-glycosylation is very different from that of monomeric serum IgA (32). SIgA has about 8 % oligomannose structures and 60 % glycans with exposed GlcNAc with less than 15 % for all glycans sialylated (32), compared to monomeric serum IgA where most of the glycans are sialylated. However, SIgA comprises only less than 1 % of total polymeric serum IgA.

We observed that polymeric serum IgA contains more IgA2 than monomeric IgA. This could be because IgA2 polymerises more easily than IgA1, or that IgA2-producing B-cells preferentially secrete polymeric IgA. Bone-marrow-derived IgA as present in serum is largely monomeric and of the IgA1 subclass, whereas mucosal IgA is largely polymeric, containing J chain and secretory component (44), and contains a substantial fraction of IgA2 (35). Therefore, an increased fraction of IgA2 and the presence of SIgA in circulating polymeric IgA may suggest its production by the mucosal immune system. Quantitative measurement of the presence of SIgA in polymeric serum IgA suggests that only a minor part of polymeric IgA contains secretory component. We hypothesize that this SIgA is derived from the mucosal surface. Circulating dimeric IgA without secretory component could be partially produced in mucosal lymphoid tissue and directly transported towards the circulation.

Although SIgA requires transepithelial transport for the attachment of secretory component to dimeric IgA, the presence of low concentrations of circulating SIgA has been described before (17,45,46). Moreover, increased serum levels of SIgA have been reported in various diseases (17,47-49). In contrast to previous studies, we now determined the SIgA concentration in highly purified polymeric serum IgA. Our data demonstrate a clear relative increase in SIgA in polymeric IgA from patients with IgAN compared with controls. We recently reported a preferential interaction of SIgA with mesangial cells, and showed glomerular accumulation of SIgA in IgAN (17). Therefore, our current results further support a role for SIgA in the pathogenesis of IgAN.

Taken together, the presented data suggest that a part of circulating polymeric IgA has a mucosal origin. There is accumulating evidence that the pathogenesis of IgAN is related to aberrant production of IgA. In this respect, in vivo studies indicated that IgAN patients have a disturbed mucosal immune response, which was restricted to production of antibodies of the IgA1 subclass (50). Our present observation that secretory IgA is increased in the polymeric IgA fraction of IgAN patients further supports a role for abnormal mucosal immunity. Since IgAN is a slowly progressive disease, it is well conceivable that only a minor subfraction of circulating IgA in IgAN patients is abnormal, and that this IgA gradually accumulates in the mesangial area. We hypothesize that this abnormal IgA is at least partially derived from the mucosal immune system. Since our data strongly indicate that large-sized IgA is especially able to interact with mesangial cells, and to induce complement activation, the gradual deposition of such pro-inflammatory IgA may eventually lead to renal disease.

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