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Pathogenic role of (S)IgA in IgA nephropathy

Oortwijn, B.D.

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CHAPTER 4

Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease

Anja Roos[1], Maria Pia Rastaldi[2], Novella Calvaresi[2], Beatrijs D. Oortwijn[1], Nicole Schlagwein[1], Danielle J. van Gijlswijk-Janssen[1], Gregory L. Stahl[3], Misao Matsushita[4], Teizo Fujita[5], Cees van Kooten[1] and Mohamed R. Daha[1]

[1]Department of Nephrology, Leiden University Medical Center, Leiden, the Netherlands 2Renal Immunopathology Laboratory, Fondazione D'Amico per la Ricerca sulle Malattie Renali, Associazione Nuova Nefrologia, c/o San Carlo Borromeo Hospital, Milano, Italy [3]Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA [4]Institute of Glycotechnology and Department of Applied Biochemistry, Tokai University, Hiratsuka, Japan [5]Department of Immunology, Fukushima Medical University, Fukushima, Japan

Summary

IgA nephropathy (IgAN) is characterized by glomerular co-deposition of IgA and complement components. Earlier studies have shown that IgA activates the alternative pathway of complement, whereas more recent data also indicate activation of the lectin pathway. The lectin pathway can be activated by binding of mannose-binding lectin (MBL) and ficolins to carbohydrate ligands, followed by activation of MBL-associated serine proteases (MASPs) and C4. We studied the potential role of the lectin pathway in IgAN.

Renal biopsies of IgAN patients (N = 60) showed mesangial deposition of IgA1 but not IgA2. Glomerular deposition of MBL was observed in 15 out of 60 cases with IgAN (25 %) and showed a mesangial pattern. All MBL-positive cases, but none of the MBL-negative cases, showed glomerular co-deposition of L-ficolin, MASPs and C4d. Glomerular deposition of MBL and L-ficolin was associated with more pronounced histological damage, as evidenced by increased mesangial proliferation, extracapillary proliferation, glomerular sclerosis and interstitial infiltration, as well as with significantly more proteinuria. IgAN patients with or without glomerular MBL deposition did not show significant differences in serum levels of MBL, L-ficolin or IgA, nor in the size distribution of circulating IgA. Furthermore, in vitro experiments showed clear binding of MBL to polymeric but not monomeric patient IgA, without a significant difference between both groups.

Together, these findings strongly point to a role for the lectin pathway of complement in glomerular complement activation in IgAN, and suggest a contribution for both MBL and L-ficolin in the progression of the disease.

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INTRODUCTION

IgA nephropathy (IgAN) is a common renal disease primarily characterized by mesangial deposition of IgA (reviewed in (1,2)). Mesangial IgA deposition induces an inflammatory process, involving mesangial proliferation, interstitial damage and proteinuria, which slowly progresses to sclerosis and end-stage renal failure in about one third of cases. Several effector mechanisms are likely to be involved in the induction of renal inflammation and damage, including direct interactions between IgA and mesangial cells (3-5) and IgA-mediated complement activation (1,6,7).

IgA deposition in IgAN is commonly associated with deposition of complement factors, most often C3, the terminal complex C5b-9, and properdin (8,9). Furthermore, increased levels of soluble split products of activated C3 have been observed in the circulation of IgAN patients (10-12). These findings suggest involvement of the alternative pathway. Indeed, in vitro studies with IgA from humans and rodents, as well as in vivo studies in rats, have indicated that IgA can directly activate the alternative pathway of complement (6,13-15).

The complement system can be activated via three pathways, the classical pathway, the alternative pathway, and the lectin pathway. These pathways converge at the cleavage of C3, followed by activation of the common terminal pathway and formation of C5b-9. The classical pathway involves binding of C1q to e.g. immune complexes, leading to generation of the C3 convertase C4b2a. The more recently discovered lectin pathway of complement is activated following an interaction of the plasma lectins mannose-binding lectin (MBL), L-ficolin or H-ficolin with their carbohydrate ligands (16-19). This leads to activation of MASPs (MBL-associated serine proteases) present in a pro-enzymatic complex with these lectins. Activated MASP-2 generates C4b2a, followed by C3 cleavage. In contrast to the classical pathway and the lectin pathway, the alternative pathway leads to activation of C3 in a C4-independent way, involving factors B and D and properdin.

Until now, the mechanism of complement activation in IgAN is still incompletely defined. Next to the presence of factors indicative for alternative pathway activation, several studies have shown glomerular deposition of C4, as well as circulating C4 activation products in a subpopulation of IgAN patients (10,20). Since in vitro studies indicated that IgA can not activate the classical complement pathway, and evidence for C4 activation in IgAN was observed in the absence of C1q deposition, activation of the lectin pathway of complement was hypothesized. Deposition of MBL in association with IgA, as a marker for lectin pathway activation, was reported in a subpopulation of IgAN patients by several authors (21-23), but these findings were questioned by others (24). Furthermore, the relation of glomerular MBL positivity with parameters of renal damage and complement activation via the lectin pathway was inconsistent between the different studies.

Recently, we reported that human polymeric serum IgA can bind to human MBL (25). This interaction involves the lectin domain of MBL, and results into activation of C4 and C3. Therefore, we hypothesize that C4 activation in IgAN may result from binding of serum MBL-MASP-2 complexes to IgA deposited in the renal mesangium. In the present study we have analyzed the possible contribution of the lectin path-

way of complement to activation of C4 and induction of renal damage in a well-defined set of renal biopsies of IgAN patients. Next to MBL, also deposition of the functionally related molecule L-ficolin was studied. The results indicate activation of the lectin pathway, involving both MBL and L-ficolin, in a subpopulation of IgAN patients, which is strongly associated with markers of disease progression.

MATERIAL AND METHODS

Patients and biopsies

Renal biopsies were selected from patients with IgA nephropathy of whom a renal biopsy was taken between January 2001 and December 2003. Patients were selected if adequate tissue was obtained for diagnostics (at least 8 glomeruli in light microscopy sections; complete immunohistology and electron microscopy examination), and if sufficient frozen material was available for additional staining after immunodiagnosis (at least 6 glomeruli in at least 15 (5 m thick) tissue sections). Cases with Henoch-Schönlein purpura, systemic lupus erythematosus, liver cirrhosis or other systemic diseases were excluded. In total, 60 biopsies were selected for evaluation.

Among selected patients, 69 % were males and 31 % females. Serum creatinine levels ranged from 44 to 972 µmol/l at the time of renal biopsy. Creatinine clearance was calculated according to the Cockroft formula (range 10-160 ml/min). Presentation at time of renal biopsy was as following: 58 % urinary abnormalities, 32 % chronic renal failure, and 10 % acute renal failure. Chronic renal failure was defined as serum creatinine value >124 µmol/l or creatinine clearance <80 ml/min in at least three determinations prior to renal biopsy and further confirmed during hospitalization. Acute renal failure was defined as an abrupt decline in glomerular filtration rate detected at patient admission, without previous altered examinations and without ultrasonographic signs of chronic renal injury.

Urine samples (24 hour collection) and serum samples were obtained at time of renal biopsy.

For additional immune fluorescence studies, a more recent series of renal biopsies was collected from IgAN patients (N = 25) using the same selection criteria as described above. From these patients, detailed clinical and histological information has not been collected.

Serological analysis

Serum IgA concentration was measured by standard nephelometric immunoassay. MBL complex activity was assessed by ELISA using coated mannan as a ligand and detecting the activation of purified C4, as described before (26) with some modifications (27). MBL complex activity was expressed in arbitrary units per ml based on the activity of a normal human pool serum. MBL concentrations were assessed by sandwich ELISA as described previously (25). Concentrations of L-ficolin were assessed using a similar protocol. In brief, plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with mAb GN5 (5 µg/ml; mouse mAb against human L-ficolin) or mAb 3E7 (5 µg/ml; mouse IgG1 anti-human MBL) in coating buffer (100 mM Na2CO3/ NaHCO3, pH 9.6). After each step, plates were washed with PBS containing 0.05 % Tween 20. Residual binding sites were blocked by incubation with PBS containing 1 % BSA. Serum samples and subsequent detection antibodies were diluted in PBS containing 1 % BSA and 0.05 % Tween 20, and incubated for one hour at 37 °C. Primary antibodies were mAb 3E7 and mAb GN5, respectively, conjugated to digoxygenin (dig) (from

Roche Diagnostics, Almere, The Netherlands). Binding of mAb was detected using HRP-conjugated sheep anti-dig antibodies (Fab fragments, from Roche). Concentrations of MBL and L-ficolin were calculated using serial dilutions of a standard serum with a known concentration of MBL (kindly provided by Dr. P. Garred, Kopenhagen, Denmark) or L-ficolin (kindly provided by Dr. D.C. Kilpatrick, Edinburgh, UK), respectively.

Purification of serum IgA and assessment of MBL binding to IgA

IgA was purified starting from 350 µl of patient serum (N = 12). The serum was directly applied to an IgA affinity column (Sepharose beads conjugated to mAb HisA43 anti-human IgA, kindly provided by Dr. J. van den Born, Amsterdam, the Netherlands), using Veronal-buffered saline containing 2 mM EDTA as a running buffer. IgA was eluted using 0.1 M Glycin / 0.3 M NaCl (pH 2.8) and fractions were immediately neutralized by 1 M Tris pH 8.0. Fractions were assessed for IgA content and the IgA peak was pooled and concentrated until approximately 350 µl. These samples were further purified on a Sephadex 200 gel filtration column (25 ml, from GE Healthcare, Munich, Germany), using the same running buffer. Fractions of 0.3 ml were collected.

On basis of the protein profile, fractions were selected that contain polymeric, dimeric and monomeric IgA, respectively. IgA concentration in these fractions was quantified by IgA sandwich ELISA. Increasing concentrations of IgA were coated on ELISA plates and binding of purified human MBL was assessed as described previously (25) using dig-conjugated mAb 3E7 for detection. In parallel, the amount of immobilized IgA was detected using biotinylated goat anti-human IgA (Dako, Glostrup, Denmark), followed by HRP-conjugated streptavidin (Zymed, Invitrogen, Breda, The Netherlands).

The relative amounts of polymeric, dimeric, and monomeric serum IgA were quantified on basis of the protein profile obtained following gel filtration of purified IgA, using calculation of the area under the curve.

Immunohistology and immunofluorescence

For immunofluorescence and immunoperoxidase stainings, the unfixed renal tissue was embedded in OCT compound (Sakura Tissue-tek, Bayer, Leverkusen, Germany), snap-frozen in a mixture of isopentane and dry-ice and stored at -80 °C. Subsequently, 5 µm sections were placed on slides and stored at -20 °C until immunostaining.

We used mouse monoclonal antibodies directed against the following molecules: MBL (mAb 3E7 (28), mAb 1C10 and mAb 2A9 (29)), L-ficolin (mAb GN4, mAb GN5 (30)), MASP-1/3 heavy chain (mAb 1E2 and mAb 4C2 (31,32)), C4 binding protein (mAb from Quidel, San Diego, CA, USA), C5b-9 (aE11, from Abcam, Cambridge, UK), IgA1 (mAb NI69-11 from Nordic Tilburg, The Netherlands (33)) and IgA2 (mAb 14-3-26 from Becton Dickinson, Erembodegem, Belgium and mAb NI512 from Nordic (33)). Rabbit polyclonal antibodies were applied for detection of C3 (FITC-labeled anti-human C3c, from Dako), C4d (from Biomedica, Vienna, Austria (34)) and MASP-2 (a MASP-2-specific antibody generated in the laboratory of Nephrology using the recombinant protease domain of MASP-2 ((35,36)) kindly provided by Dr. P. Gal, Institute of Enzymology, Budapest, Hungary; to be described elsewhere).

For indirect immunofluorescence, after fixation in cold acetone, tissues were incubated sequentially with the primary antibody and the proper fluorescently labeled secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse Ig or goat anti-rabbit Ig, Molecular

Probes). Slides were finally mounted with an anti-fading aqueous mounting medium (Fluorsave, Calbiochem).

Immunoperoxidase staining of MBL was performed as previously described (37). Briefly, after incubation with 0.5 % avidin (Sigma Chimica, Milan, Italy) and 0.01 % biotin (Sigma), to suppress endogenous avidin-binding activity, tissue sections were fixed in a methanol-H2O2 solution to block endogenous peroxidase. After washing, sections were incubated with primary antibody, followed by biotinylated anti-mouse antibody (Zymed), and peroxidase-labeled streptavidin (Zymed). Peroxidase activity was detected with 3,5-diaminobenzidine (DAB, from Sigma), sections were counterstained with Harry's hematoxylin, dehydrated, and mounted in Permount.

In both immunofluorescence and immunoperoxidase methods, specificity of antibody labeling was demonstrated concurrently using proper control immunoglobulins (Zymed).

Evaluation of renal tissue

Evaluation of renal tissue was performed blindly by two independent observers. For immunostaining, tissues were scored as negative (0) or positive (1), according to the detection of staining in the majority of glomeruli, in at least 3 tubular cross-sections per field, and in vessel endothelium.

For histology, sections were stained using standard periodic-acid Schiff (PAS), periodic-acid-silver methenamine (PASM), and/or Trichrome techniques. Mesangial proliferation was scored as 1+ when mild-moderate (i.e. between 4 and 6 cells per mesangial area) and 2+ when intense (more than 6 cells per mesangial area). Extracapillary proliferation, global sclerosis, and segmental sclerosis were calculated as percentage of the total number of glomeruli. Interstitial infiltration and fibrosis were scored 0 when absent, 1+ when mild (involving < 30 % of the interstitium), 2+ when moderate (30-60 % of the interstitium involved), or 3+ when intense (when present in > 60 % of the renal interstitium). Hyalinosis of the vessel wall was indicated when absent (0) or present (1).

Statistical analysis

Data were compared between IgAN patients showing positive and negative glomerular staining for MBL, respectively. Frequency analysis was performed using Chi-square test. Other comparisons were evaluated using the Mann Whitney U test. The Spearman Rank correlation coefficient was used to analyze correlation. Differences were considered statistically significant when P was below 0.05.

RESULTS

Evidence for glomerular lectin pathway activation in IgA nephropathy

The presence of MBL was examined in 60 renal biopsies from IgAN patients. Glomerular staining for MBL was observed in a predominantly mesangial pattern in 15 biopsies (25 %, Figure 1A, C) whereas glomeruli in 45 IgAN biopsies stained negative for MBL (Figure 1D). A similar positive staining was observed using three different mAb directed against MBL, whereas staining with an isotype control mAb was completely negative (Figure 1B).

Next we examined the presence of other molecules involved in the lectin pathway of complement. All renal biopsies with positive glomerular staining for MBL also

showed glomerular staining for L-ficolin, as shown by two different mAb, whereas glomerular L-ficolin staining was negative in all MBL-negative cases (Table 1, Figure 2). Similarly, all cases positive for MBL and L-ficolin, but none of the negative cases, showed positive mesangial staining for the common heavy chain of MASP-1 and MASP-3, using two mAb, and for C4d and C4 binding protein (Table 1, Figure 2). The majority of biopsies in both MBL-negative and MBL-positive groups showed deposition of C3, whereas C1q was negative (Table 1).

All IgAN biopsies showed a typical mesangial deposition of IgA1 (Table 1, Figure 2). In contrast, we were unable to show a positive staining for IgA2, using two different IgA2-specific mAb (Table 1, Figure 2), although these antibodies showed clear positive staining on renal biopsies from lupus nephritis patients (data not shown).

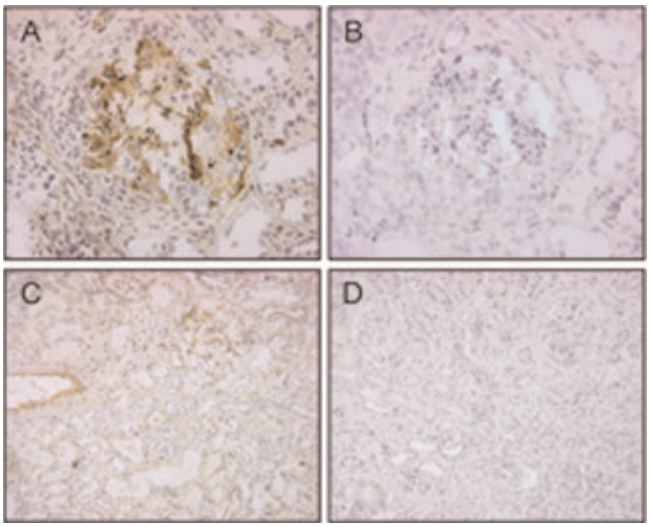

Figure 1. Glomerular MBL deposition in IgAN patients. *Biopsies from patients with IgAN were stained with mAb 1C10 (A), mAb 3E7 (C, D) or an isotype control mAb (B). Figures A, C, and D are derived from different patients who showed positive (A, C) or negative (D) staining for MBL. Figures A and B are from the same patient. Please note tubular and vascular staining for MBL in C, in addition to glomerular staining.*

In order to further extend the analysis of complement activation in IgAN, deposition of MASP-2, being the C4-cleaving enzyme of the lectin pathway, and C5b-9, as the final product of the terminal pathway of complement, was evaluated. Due to a shortage of renal tissue, an additional series of 25 renal biopsies was used for these studies. From these biopsies, 6 showed positive glomerular staining for MBL (24 %), confirming the results presented above in an independent study. All MBL-positive biopsies showed positive glomerular staining for MASP-2 (Figure 2), whereas MASP-2 was negative in all other biopsies. Staining for C5b-9 was clearly positive in a mesangial pattern in all IgAN biopsies. However, in MBL-positive biopsies, staining was observed with a high intensity (Figure 2), whereas intensity showed a marked variation in MBL-negative biopsies (not shown).

Glomerular lectin pathway activation in IgA nephropathy is associated with markers of disease progression

Data indicated above show that IgAN patients can be divided in two groups, with (25 %) and without (75 %) evidence for glomerular activation of the lectin pathway of complement. These two patient groups were further characterized.

MBL-positive and negative cases had a similar male/female distribution and a similar age at the time of the renal biopsy (Table 2). However, patients with glomerular MBL-positivity presented twice as often with renal failure at the time of renal biopsy, mostly chronic renal failure ($P < 0.0001$, Table 2). Furthermore, MBL-positive cases showed significantly more proteinuria than MBL-negative IgAN patients, whereas, in contrast, less MBL-positive IgAN patients suffered from episodes of macroscopic hematuria (Table 2, Figure 3A, B). In our IgAN patient population, macroscopic hematuria seems to be associated with a more benign disease course,

Table 1. Molecular composition of glomerular deposition in IgA nephropathy*

MBL staining	L-ficolin	MASP-1/3	C4d	C4 binding protein	C1q	C3	IgA1	IgA2
Neg (N = 45)	0%	0%	0%	0%	0%	82%	100%	0%
Pos (N = 15)	100%	100%	100%	100%	0%	60%	100%	0%

*The data indicates the percentage of biopsies with positive staining for a certain marker. Positive staining means clear positivity in the majority of glomeruli. Neg: negative, Pos: positive

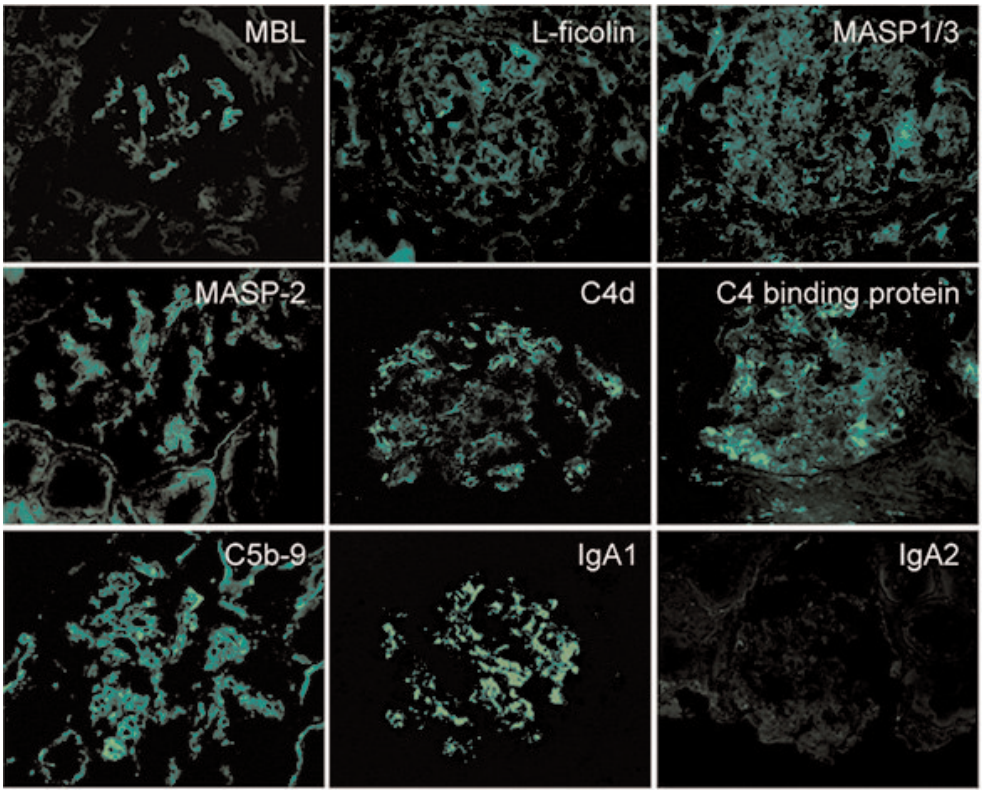

Figure 2. Lectin pathway activation in IgAN.
Renal tissue from IgAN patients was stained for the presence of MBL (mAb 3E7), L-ficolin (mAb GN4), MASP-1/3 (mAb 1E2), MASP-2, C4d, C4 binding protein, C5b-9, IgA1 and IgA2 (mAb NI512), as indicated. Representative images are shown.

since patients with macroscopic hematuria showed significantly less proteinuria than patients without macroscopic hematuria, also when MBL-negative cases were analysed only (P = 0.04, not shown). MBL-positive cases also showed a higher serum creatinine level than MBL-negative IgAN patients, although the creatinine clearance was not significantly different (Table 2). However, the latter parameter could be analyzed for only part of the patients in our study.

Data presented above suggest that glomerular MBL-positivity in IgAN is associated with a more severe disease. This is strongly supported by semi-quantitative histological evaluation of renal damage, demonstrating that biopsies from IgAN patients with positive glomerular staining for MBL had more intense mesangial proliferation as well as significantly more extracapillary proliferation, global sclerosis and interstitial infiltration than MBL-negative cases (Figure 3C-F, Table 3). Increased glomerular and tubulointerstitial damage in MBL-positive cases, as compared to MBL-negative cases, is illustrated in Figure 4.

Table 2. Clinical and laboratory data from IgA nephropathy patients

Parameter	MBL-negative	N	MBL-positive	N	P
Age at renal biopsy (yr;median)	35	36	27	12	0.28
Female gender (%)	36	36	17	12	0.21
Renal failure (% chronic)	33 (73 % chronic)	45	67 (80 % chronic)	15	**<0.0001**
Proteinuria (gram/24 hr;median)	0.7	45	2.5	15	**0.004**
Macroscopic hematuria present (%)	46	39	13	15	**0.025**
Serum creatinine (µmol/l;median)	97	45	133	15	**0.042**
Creatinine clearance (ml/min;median)	92	25	61	10	0.43
Serum MBL (ng/ml;median)	1299	28	873	12	0.8
MBL complex activity (U/ml;median)	738	29	865	12	0.27
Serum L-ficolin (µg/ml;median)	2.6	28	1.82	12	0.15
Serum IgA (mg/ml;median)	2.54	36	2.28	15	0.37
Monomeric serum IgA (%;median)	73	6	73	6	0.7
Dimeric serum IgA (%;median)	23	6	20	6	0.39
Polymeric serum IgA (%;median)	4	6	5.4	6	0.09

MBL-negative cases and MBL-positive cases are defined on basis of glomerular staining. The size distribution of purified serum IgA is expressed as % of total IgA. All data were obtained at the time of the renal biopsy.

No difference between the two groups was found for the presence of vascular lesions. Glomerular staining for MBL was significantly associated with staining for MBL in vessels and tubuli (Table 3 and Figure 1C). However, tubular and vascular staining for MBL was only weakly associated with mesangial proliferation (P = 0.03), and no association was found with proteinuria or other parameters of disease activity. Furthermore, tubulointerstitial staining for MBL was not clearly associated with markers of complement activation (not shown).

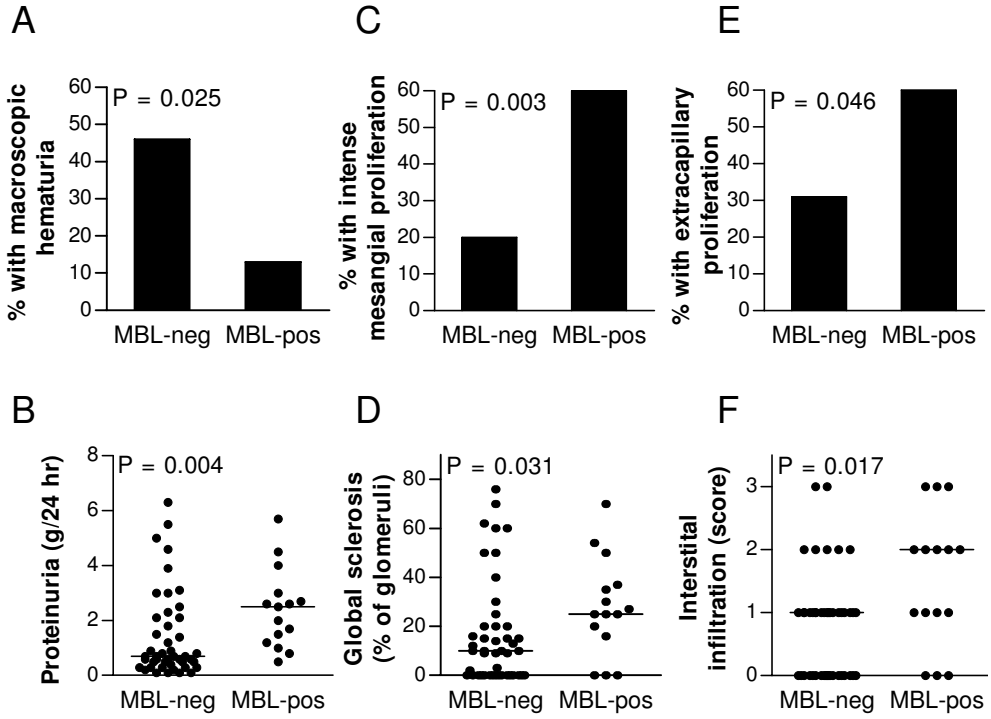

Figure 3. MBL positivity is associated with markers of renal damage.
IgAN patients were divided in two groups on basis of glomerular positivity for MBL. Clinical (A, B) and histological data (C-F) presented are scored as explained in Materials and Methods. Horizontal lines in B, D, and F represent the median. Statistical analysis was performed as described in Tables 2 and 3.

Table 3. Histological data from IgA nephropathy patients

Parameter	MBL-neg (N = 45)	MBL-pos (N = 15)	P
Intense mesangial proliferation (% of cases)	20	60	**0.003**
Extracapillary proliferation present (% of cases)	31	60	**0.046**
Global sclerosis (% of glomeruli;median)	10	25	**0.031**
Segmental sclerosis (% of glomeruli;median)	0	8	0.069
Interstitial infiltration (0-3 scale scoring;median)	1	2	**0.017**
Interstitial fibrosis (0-3 scale scoring;median)	1	2	0.12
Vessel lesions present (% of cases)	33	40	0.64
Vascular MBL staining positive (% of cases)	36	73	**0.01**
Tubular MBL staining positive (% of cases)	22	80	**0.0001**

MBL-negative cases and MBL-positive cases are defined on basis of glomerular staining

Characterization of circulating MBL, L-ficolin and IgA

We further examined whether the observed dichotomy in IgAN patients with respect to glomerular lectin pathway activation could be associated with certain properties of the lectin pathway of complement and /or the IgA in the circulation.

Serum levels of MBL are genetically determined and highly variable in the human population (27). A similar variability was observed for IgAN patients in our study, without a significant difference between cases showing positive and negative staining for MBL (Figure 5A and Table 2). Serum levels of L-ficolin showed less variability, and also did not distinguish between both groups (Figure 5B and Table 2). As a functional parameter, we assessed MBL complex activity, being a measure of the ability of the MBL-MASP-2 complex to activate C4. MBL complex activity showed a similar distribution in both groups (Figure 5C and Table 2), and was strongly correlated to MBL serum concentration (Figure 5D). As expected, three patients with clear MBL deficiency (Figure 5D) showed negative glomerular staining for MBL.

Since these data exclude the simple explanation that lectin pathway deficiency explains the lack of glomerular lectin pathway activation in most IgAN patients, we further studied whether properties of serum IgA may explain the observed dichotomy. The concentration of serum IgA was not significantly different between both groups (Figure 5E and Table 2). To address the question more in depth, serum IgA was purified from serum from IgAN patients. From patients with and without glomerular MBL deposition, we selected 6 patients in each group. Patients with a (possible) MBL deficiency were excluded. IgA was purified from serum and subjected to gel filtration. The protein profile clearly reveals the presence of polymeric,

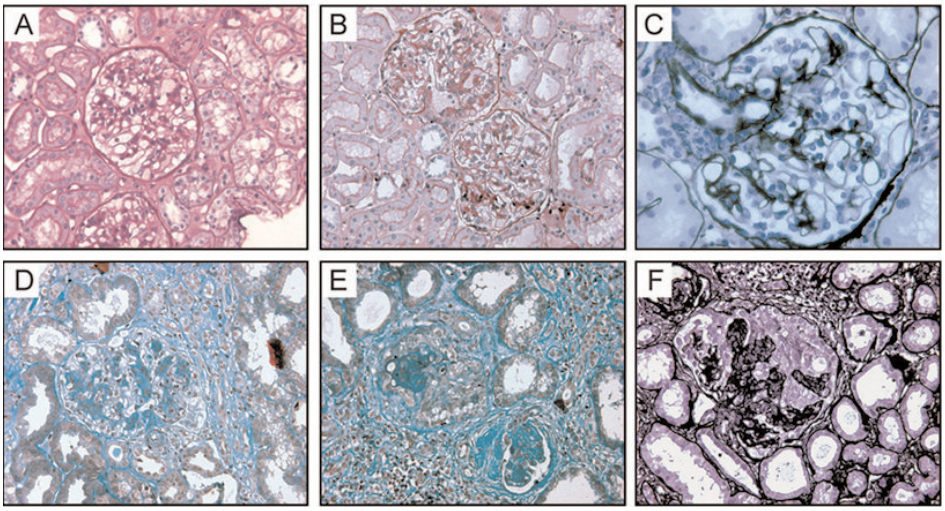

Figure 4. Renal histological damage in IgAN patients.
Renal histology from three patients without glomerular MBL deposition (A-C) shows glomeruli affected by mild mesangial proliferation and mild mesangial matrix expansion. No major tubulo-interstitial lesions are visible. Images D-F represent patients with glomerular MBL deposition (N = 3). Glomerular injury is characterized by intense mesangial proliferation and mesangial matrix expansion (D), segmental and global sclerosis (E), and extracapillary proliferation (F), whereas tubular dilations, interstitial infiltration and fibrosis are evident in the interstitium (D-F). Sections were stained with PAS (A, B), PASM (C, F) or trichrome (D, E) techniques.

dimeric and monomeric IgA (Figure 6A). The size distribution of serum IgA in IgAN patients with and without glomerular MBL deposition showed a low variability without any significant differences (Figure 6B and Table 2).

Subsequently, we studied the binding of purified MBL to patient IgA of different sizes. Therefore, increasing concentrations of monomeric, dimeric, and polymeric IgA were coated on ELISA plates, and binding of MBL was assessed. The results showed a high variability in the MBL binding properties of IgA from different donors, although similar amounts of IgA were immobilized on the ELISA plates (Figure 7A). Monomeric IgA did not show MBL binding, whereas strong but donor-dependent MBL binding could be observed to polymeric IgA (Figure 7B). MBL binding to dimeric IgA was significantly less than that to polymeric IgA ($P < 0.01$). Quantification of the binding of MBL per unit immobilized IgA did not reveal a significant difference between patients with and without glomerular MBL positivity, respectively (Figure 7B). However, two patients whose IgA showed strong MBL binding both showed positive deposition of MBL in the glomerulus.

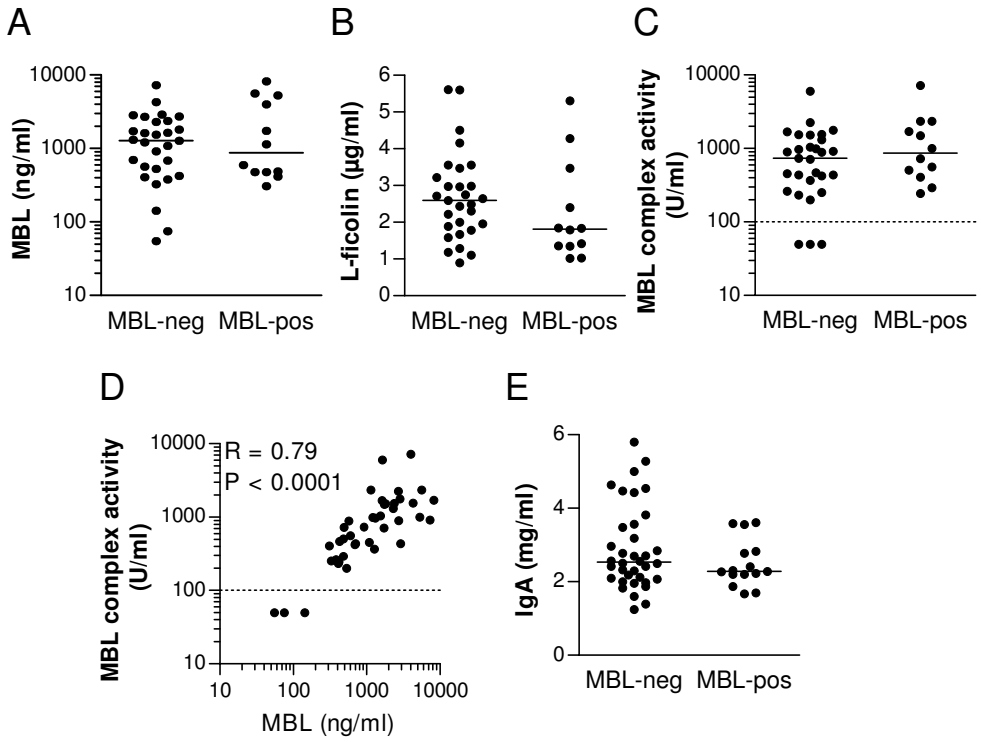

Figure 5. Circulating MBL, L-ficolin, and IgA in IgAN patients. *Concentrations of MBL (A), L-ficolin (B) and IgA (E), as well as MBL complex activity (C) were quantified in serum of IgAN patients as described in Materials and Methods. IgAN patients were divided in two groups on basis of glomerular positivity for MBL. Horizontal solid lines represent the median, and dashed lines indicate the detection limit. Correlation between MBL concentration and MBL complex activity is shown in D.*

DISCUSSION

Data from the present study indicate that IgAN patients can be divided in two groups on the basis of the pattern of complement activation. About 75 % of IgAN patients show negative glomerular staining for MBL, L-ficolin, MASP, C4d and C4-binding protein, indicating that C3 and C5b-9 activation in these patients most likely occurs via the alternative pathway. In contrast, 25 % of IgAN patients show glomerular deposition of MBL, L-ficolin, MASP, C4d but not C1q, which is strongly indicative of activation of complement via the lectin pathway of complement. Importantly, the clinical and histological data clearly indicate that activation of the lectin pathway of complement is associated with more severe renal damage.

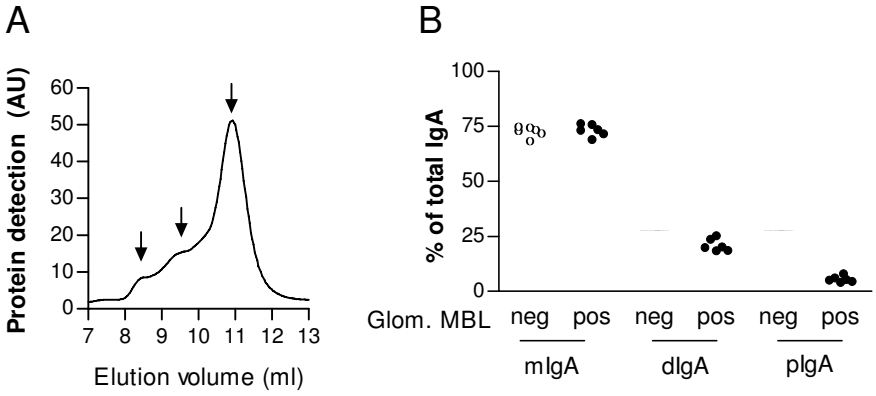

Figure 6. Size distribution of circulating IgA. *A. Typical example of a protein profile obtained after gel filtration of purified IgA. Position of polymeric, dimeric and monomeric IgA is indicated. B. Quantification of IgA size distribution of IgA from 12 IgAN patients showing positive or negative glomerular staining for MBL.*

Using a well-defined polyclonal antibody to C4d, which is now frequently used in the diagnostic evaluation of renal transplant rejection (34), we observed deposition of glomerular C4d in IgAN exclusively in association with molecules of the lectin pathway. An identical pattern was observed for deposition of C4-binding protein, a regulatory protein that was also previously reported as a sensitive marker for the presence of C4 (20). Activated C4 is most likely generated by MASP-2, the key complement-activating enzyme of the lectin pathway, which was shown to be present in the mesangial area in association with MBL.

Next to strongly suggesting activation of C4 via the lectin pathway in a subpopulation of IgAN patients, our data indicate glomerular activation of C3 and C5b-9 via the alternative pathway in the majority of IgAN patients, independently of C4. The latter aspect is in agreement with a number of in vitro studies showing alternative pathway activation by IgA from human and rodent species (6,13,15).

We assume that mesangial MBL and L-ficolin are derived from the circulation as preformed complexes with MASPs, although local production of molecules of the lectin pathway can not be excluded (38). Our previous in vitro studies showed that MBL is able to interact via its lectin domain with purified polymeric serum IgA (25).

Further support for an interaction between MBL and IgA is provided by studies in Henoch Schönlein Purpura nephritis (39,40). In the present study, we show that MBL binds to polymeric IgA from IgAN patients. It is possible that MBL binds to glycans present on the IgA heavy chains. IgA consists of two subclasses, IgA1 and IgA2, of which the former dominates in the circulation (90 % IgA1). However, mucosal IgA may contain up to 65 % IgA2 (7). Interestingly, circulating IgA1 from IgAN patients was reported to have aberrant glycosylation of O-linked glycans, potentially involved in recognition by lectins (41).

Although it is generally believed that IgA deposits in IgAN consist almost exclusively of IgA1 (1,2,7,33,42,43), biopsies from IgAN patients may also contain IgA2 (44). A recent study in this field by Hisano et al. (23) showed that about 50 % of a

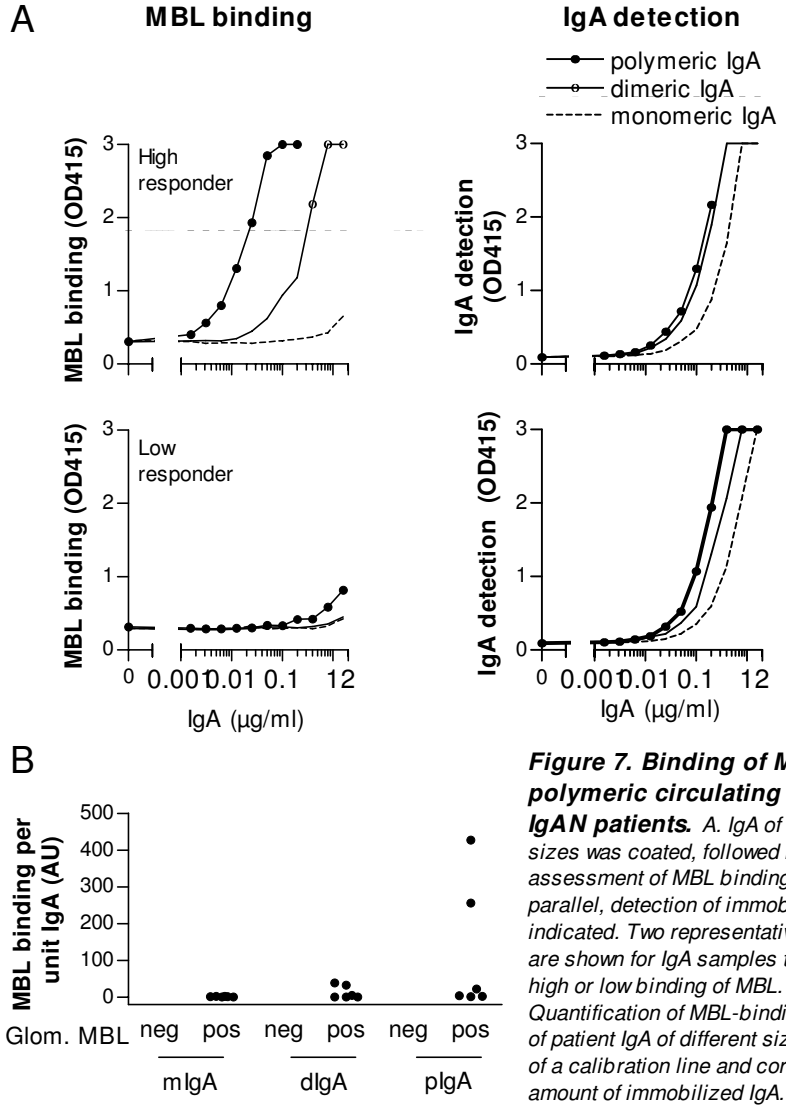

Figure 7. Binding of MBL to polymeric circulating IgA from IgAN patients. *A. IgA of different sizes was coated, followed by assessment of MBL binding and, in parallel, detection of immobilized IgA, as indicated. Two representative examples are shown for IgA samples that show a high or low binding of MBL. B. Quantification of MBL-binding properties of patient IgA of different sizes, on basis of a calibration line and corrected for the amount of immobilized IgA.*

Japanese population of IgAN patients showed deposition of IgA2 next to IgA1, whereas glomerular MBL deposition was exclusively observed in these IgA2-positive patients. Similar results were recently reported in Henoch-Schönlein purpura nephritis (40). Stimulated by these striking observations, we investigated the glomerular presence of IgA2 in our patient population, using two different IgA2-specific mAb, including mAb 14-3-26 that was also used by Hisano et al. (23). However, we were not able to detect any glomerular positivity for IgA2 in IgAN biopsies. This difference might be due to ethnical differences between patient populations. Furthermore, although we tried several amplification methods to acquire a signal for IgA2, it could still be that IgA2 is present in tiny amounts and/or masked by other molecules, thereby hampering detection.

IgA deposited in the renal mesangium of IgAN patients is classically accepted as being polymeric IgA (45). Polymeric IgA contains several other molecular components, which are currently only partially identified (45,46). It can not be excluded that MBL binds to a glycosylated molecule associated with polymeric IgA and present in the mesangial deposits of IgAN patients. An alternative explanation would be that MBL binds to injured tissue in the glomerulus, which is in line with the binding of MBL to apoptotic and necrotic cells (47).

To our best knowledge, data from the present study are the first indication for a role of L-ficolin in disease. In vitro data indicate that ligand specificities of MBL and L-ficolin do only partially overlap (16,17,48). However, recent experiments in our group indicate that L-ficolin also binds to polymeric IgA (Roos et al. manuscript in preparation). MBL and ficolins have an important role in innate immune defense. However, evidence is increasing that MBL and the lectin pathway of complement can also be harmful for the host as mediators of inflammation. In this respect, MBL has been proposed to be involved in ischemia/reperfusion injury (49), diabetic nephropathy (50) and ulcerative colitis (51). In the present study, we observed that IgAN patients with glomerular MBL deposition and lectin pathway activation showed significantly more renal damage, as evidenced by increased mesangial proliferation, extracapillary proliferation, glomerular sclerosis, interstitial infiltration, and proteinuria. An association of glomerular MBL positivity with decreased renal function and increased mesangial proliferation and proteinuria was also reported by Matsuda et al. (21) but could not be confirmed by studies from Endo et al. (22) and Hisano et al. (23). In Henoch-Schönlein purpura nephritis, MBL deposition was associated with increased progression of renal disease (40). Increased disease progression in association with glomerular lectin pathway activation may possibly be ascribed to pro-inflammatory activation products of the complement system, including C5a and C5b-9, and potentially also to a direct effect of MBL.

Similar to several other studies (21-23), we observed lectin pathway activation only in a subpopulation of IgAN patients. The majority of IgAN patients without mesangial MBL deposition did not show any evidence for deficiency of MBL or L-ficolin. Interestingly, serum concentrations of MBL in this population of IgAN patients (N= 41, median = 1213 ng/ml) were significantly higher than those in a population of Caucasian healthy controls (N = 190; median = 677 ng/ml, P = 0.002, data not shown). This difference could be explained by genetic differences and/or by conditions associated with renal disease.

We hypothesize that the difference between IgAN patients with and without mesangial MBL deposition is based on differential availability of the ligand, potentially related to differences in IgA glycosylation. In order to further examine this hypothesis, we investigated the binding of purified MBL to polymeric, dimeric and monomeric IgA from IgAN patients being either positive or negative for glomerular MBL deposition. This analysis revealed binding of MBL to polymeric serum IgA with a large inter-individual variation, but the degree of MBL binding could not be directly related to glomerular lectin pathway activation. However, mesangial IgA is not necessarily similar to serum IgA, and furthermore, it could be that glomerular IgA is differentially accessible in IgAN patients. We assume that the observed dichotomy in IgAN patients is due to several factors acting in combination.

In conclusion, the current study shows that MBL and the lectin pathway of complement are involved in complement activation in a subpopulation of patients with IgAN, which is highly likely to play an adverse role in the disease. In vitro data point to a role for polymeric IgA in lectin pathway activation. Precise identification of the ligand for MBL and L-ficolin in the mesangium, which is presumably present and/or accessible in only part of the patients with IgAN, will provide novel insight in the pathogenesis of IgAN and may provide novel therapeutic options to treat disease progression.

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