# Pathogenic role of (S)IgA in IgA nephropathy

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CHAPTER 6

# A pathogenic role for Secretory IgA in IgA Nephropathy.

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**Summary**

IgA nephropathy (IgAN) is characterized by deposits of IgA in the renal mesangium. It is thought that deposits of IgA mainly involve high molecular weight (HMW) IgA1. However there is limited information on the exact composition of HMW IgA in these deposits. In this study we investigated the presence of secretory IgA (SIgA) in human serum and in the glomerular deposits of a patient with IgAN. Furthermore, we analysed the interaction of SIgA with mesangial cells. With ELISA SIgA concentrations in serum of IgAN patients and healthy controls was measured. Patients and controls both have circulating SIgA that was restricted to the HMW fractions. Patients tend to have higher levels of SIgA, but this difference was not significant. However, in patients with IgAN, high serum SIgA concentrations were associated with hematuria. Binding of size fractionated purified serum IgA and secretory IgA to mesangial cells was investigated with flow cytometry. These studies with mesangial cells showed stronger binding of SIgA to primary mesangial cells, compared to binding of serum IgA. Importantly, after isolation and elution of glomeruli from a nephrectomized transplanted kidney from a patient with recurrent IgAN, we demonstrated a 120-fold accumulation of SIgA compared to IgA1 in the eluate. In conclusion we have demonstrated that SIgA strongly binds to human mesangial cells, and is present in significant amounts in serum. Furthermore, we showed that SIgA is accumulated in the glomeruli of an IgAN patient. These data suggest an important role for SIgA in the pathogenesis of IgA nephropathy.

*Kidney Int. 69: 1131, 2006*

## INTRODUCTION

Primary IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis. The disease shows a spectrum of clinical presentations, leading to progressive renal failure in a substantial proportion of patients. The hallmark of this disease is deposition of IgA in the glomerular mesangium (1-3). It is generally thought that deposits of IgA mainly involve IgA1 and for a large part consist of high molecular weight (HMW) IgA (4). The composition of HMW forms of IgA in serum is diverse and may include dimeric IgA, CD89/IgA complexes, IgA immune complexes and IgA-fibronectin complexes (5-9).

Several reports have shown that the glycosylation of IgA1 in patients is different from that in controls. Patient IgA1 contains more terminal GalNAc and this could play a role in the deposition of IgA1 in the mesangium (10-12). IgA from the glomeruli of IgAN patients consists at least partly of HMW IgA (13) and is under-O-glycosylated (10,14). In these studies no data was presented concerning the presence of secretory IgA (SIgA). When deposited in the kidney, the IgA1-containing complexes are linked to inflammation. Stimulation of mesangial cells with HMW IgA leads to enhanced production of cytokines and chemokines, including IL-6, TGF-β, TNF-α, MCP-1, IL-8 and MIF (15-18). These cytokines and chemokines might play a role in the development and progression of renal injury in IgAN patients.

SIgA is the dominant immunoglobulin in external mucosal secretions like in oral, respiratory, and intestinal cavities, and is often characterized as a component of the immune systems "first-line defence" against pathogenic micro-organisms (19). The SIgA molecule is composed of two IgA monomers, linked by a junction peptide called J chain, and the secretory component (SC) that wraps around the dimer (20). Next to its presence in mucosal secretions, small amounts of SIgA can also be found in human serum (21,22). Moreover, increased serum levels of SIgA have been reported in various diseases (23-25), indicating that SIgA may be a marker of clinical interest. A previous study has suggested that the serum concentrations of SIgA are not different in IgAN patients compared to healthy controls (23). The physiological roles of serum IgA and SIgA are quite different, and the presence of the highly glycosylated SC can have major effects on the biological functions of SIgA (26,27). In literature it is still controversial if SIgA is able to bind to mesangial cells (28,29). One study showed that SIgA is able to bind to mesangial cells (28), however this could not be confirmed in another study (29).

In the present study we investigated the presence of SIgA in sera of IgAN patients and healthy controls, and examined the binding of different molecular forms of IgA to human mesangial cells with special interest for SIgA. Finally we investigated the presence of SIgA in the glomerular eluate of an IgAN patient.

## MATERIAL AND METHODS

### Human subjects

In this study, we included 19 healthy volunteers and 47 patients with primary IgAN (30). The latter were defined by mesangial deposits of IgA. None of these patients had clinical or laboratory evidence of Henoch Schoenlein purpura, systemic lupus erythematosus, liver disease or received immunosuppressive therapy. Patients were included in the study between

October 1998 and February 1999. Blood and urine samples were collected, clinical characteristics and laboratory data of the patient group were obtained (Table 1) retrospectively (observation time 5.9 ± 0.5 years) and prospectively (follow-up time 3.7 ± 0.2 years).

Renal cortex was obtained from a transplant nephrectomy from a male patient (1977), with biopsy-proven recurrent IgAN. He was diagnosed with macroscopic hematuria (serum creatinine 146 µmol/l) in 1993. The renal biopsy of 1996 showed characteristic features of progressive IgAN, and dialysis was started. In April 2002 he received a cadaveric renal transplantation. Three months later a decline in renal function was observed and a renal biopsy showed the presence of interstitial nephritis and recurrent IgAN. In a biopsy taken 6 months following transplantation characteristics of the interstitial nephritis were disappeared, but IgA was still present. Due to decreased patient compliance there were two episodes of acute rejection in 2003 and 2004, which eventually led to graft loss. Informed consent was obtained from all subjects.

***Table 1: Clinical characteristics of the patients with IgAN at the point of serum SIgA measurement.***

| | Number of patients | Median | Range |
|---|---|---|---|
| Male/ female | 38/ 9 | | |
| ACE-inhibitor/AII antagonist | 24/ 3 | | |
| Age (years) | | 47 | 19-69 |
| Systolic blood pressure (mmHg) | | 130 | 100-160 |
| Diastolic blood pressure (mmHg) | | 80 | 55-100 |
| Proteinuria (g/day) | | 0.6 | 0.1-5.2 |
| Glomerular filtration rate (ml/min) | | 71 | 15-137 |

**IgA purification**

Serum from healthy controls was used for IgA purification, according to methods described before (9). In brief, serum was applied to an anti-IgA (HisA 43, kindly provided by dr J. van den Born, Free University Medical Center, Amsterdam) affinity column. IgA was eluted with 0.1 M glycine/ 0.3 M NaCl (pH 2.8). The eluted protein fractions containing IgA, as assessed by ELISA (31), were pooled and dialysed. The IgA was size-separated with a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column (120 ml, Amersham Pharmacia, Roosendaal, The Netherlands) into pIgA and mIgA.

**Cell culture**

NHMC (normal human mesangial cells, Cambrex, USA) were expanded according to the protocol provided by the manufacturer. Experiments with NHMC were performed in RPMI with 10 % FCS, 1 % non-essential amino acids, 0.5 % transferrin/insulin/selenium, 1 % sodium pyruvate, 1 % L-glutamine (all purchased at Gibco/ life Technologies, Paisley, Scotland). AMC11, a spontaneously growing adult human mesangial cell line (kindly provided by Prof. Holthofer, Helsinki), was cultured in DMEM with 10 % FCS. Cells were cultured in culture flasks (Greiner, Frickenhausen, Germany) at 37 ºC in a humidified incubator with 5 % CO2/ 95 % air. For passage the cells were harvested by trypsinization (0.02 % (w/v) EDTA/ 0.05 % (w/v) trypsin in PBS from Sigma (St. Louis, MO)).

Monocyte-derived dendritic cells were generated as described before (32).

**Flow cytometry**

Cells were harvested, washed with FACS buffer (0.5 x PBS containing 1 % BSA/ 2.8 % glucose/ 0.01 % $NaN_3$) and incubated with monomeric and polymeric serum IgA, and SIgA (Sigma). Following incubation for 1 hour at 4 ºC, cells were washed and incubated for one hour at 4 ºC with monoclonal anti-IgA Ab 4E8 (IgG1) (31). IgA binding was visualized using PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) and assessed for fluorescence intensity by flow cytometry (FACSCalibur, Cell quest software; BD Biosciences). Dead cells, identified by propidium iodide uptake, were excluded from analysis.

For analysing the presence of the mannose receptor, anti-mannose receptor antibody (D547.3; kindly provided by F. Koning, Leiden University Medical Center, Leiden, The Netherlands) was used followed by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark). For the detection of the two chains of MAC-1, anti-human CD11b (leu-15-PE, BD Biosciences) and anti-CD18 (IB4, ATCC) were used.

For inhibition of the binding of SIgA to mesangial cells, the cells were pre-incubated with purified free SC (100 µg/ml) (33), 10 mM EDTA or 10 mM $CaCl_2$ for 1 hour at 4 ºC. Subsequently, without washing, SIgA was added and this binding was visualized as described above. Inhibition of binding of SIgA was also detected with a pre-incubation of IgA for one hour at 4 ºC; subsequently Alexa-conjugated SIgA was added. After one hour the binding of Alexa-conjugated SIgA was measured.

**Cytokine analysis**

Production of IL-6 was measured in supernatants of mesangial cells after 72 hr stimulation. Prior to stimulation, cells were transferred to 48-wells plates (Costar, Corning, NY) at a density of 25 x $10^3$ cells per well and cultured overnight in culture medium with 0.5 % serum. Cells were stimulated with IL-1, serum IgA, or SIgA. The concentration of IL-6 in culture supernatants was measured by specific ELISA as described previously (34).

**RNA extraction and RT-PCR**

Total RNA was extracted from mesangial cells using RNeasy mini kit (Qiagen, Valencia, CA). OD260/280 ratio was measured to determine the quantity and purity of RNA preparations. Fixed amounts of total cellular RNA (1 µg) were reverse transcribed into cDNA by oligo(dT) priming, using M-MLV reverse transcriptase (Gibco/Life Technologies, Breda, The Netherlands). PCR to detect the human mannose receptor was performed with specific primers (sense 5'-TTG AGT GGA GTG ATG GGA CC-3'; antisense 5'-TTT CTG GAC CTT GGC TTC GT-3') using AmpliTaq DNA polymerase (Applied Biosystems, Roche, Mannheim, Germany). The PCR reaction was performed under standard conditions (35). The cDNA samples were also subjected to PCR for GAPDH as an internal control (35), PCR products were resolved on 1 % agarose gels and bands were visualized by ethidium bromide staining.

**Preparation of glomerular eluate**

Glomeruli were isolated from a nephrectomized kidney from a transplanted IgAN patient with recurrent disease. For this purpose the renal cortex was separated from the medulla. After slicing the cortex in little pieces, the glomeruli were collected on a 150-mesh sieve and stored at -70ºC with protease inhibitors (Complete, Mini, and Roche). The glomeruli were washed with PBS and the final pellet was resuspended in 5 ml of elution buffer (2 M KSCN in 0.01 M phosphate buffer, pH 7.6) as described before (36). After stirring at room tempera-

ture (RT) for 60 min, the suspension was centrifuged at 8,000 g for 15 min at 4ºC in a high-speed centrifuge (Beckman, Avanti J25-1). The supernatant was collected and dialysed overnight against PBS. The precipitate that was formed during dialysis was removed by centrifugation for 15 min at 17,000 g and was negative for immunoglobulins. The remaining supernatant was concentrated to one-third of the original volume.

**ELISA for human SIgA, IgA1, IgA2, IgG and IgM**

To test the specificity of the antibodies used for the SIgA ELISA, Ninety-six well Nunc Maxisorp microtitre plate (Gibco/Invitrogen) was coated with 2 µg/ml IgA and BSA in carbonate buffer (pH 9.6) overnight at RT. After washing, the plate was incubated with monoclonal antibodies (2 µg/ml) specific for secretory component: NI194-4 (IgG1-k; Nordic Immunology, Tilburg, The Netherlands) (37) or 3F8 (kindly provided by dr R.M. Goldblum, University of Texas Medical Branch, Galveston) (38) in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. Bound antibody was detected with goat anti-mouse Ig HRP (Dako). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. at 415 nm was measured using a microplate biokinetics reader (EL312e, Biotek Instruments, Winooski, Vermont, USA).

In order to quantify SIgA levels in isolated IgA and serum, a sandwich ELISA specific for SIgA was developed. Plates were coated with 2 µg/ml monoclonal antibody specific for secretory component (NI194-4; 3F8) in carbonate buffer (pH 9.6) (100 µl/well) overnight at RT. Subsequently, the plates were washed with PBS/ 0.05 % Tween. Plates were incubated with IgA or with serum from patients and controls in PBS/ 1 % BSA/ 0.05 % Tween for one hour at 37 ºC. After washing, bound IgA was detected using mouse anti-human IgA (4E8) conjugated to digoxigenin (Dig), followed by $F(ab)_2$ anti-Dig antibodies conjugated to HRP (Roche). Enzyme activity of HRP was developed using ABTS (Sigma). The O.D. at 415 nm was measured. A calibration line was produced using purified SIgA (Sigma).

Concentrations of IgA1, IgA2, IgG and IgM in sera and glomerular eluate were determined using specific ELISAs (39).

**Western blot analysis**

IgA preparations were subjected to 10% SDS-PAGE under reducing conditions, followed by semi-dry blotting to PVDF (immobilin-P, Millipore, Bedford, MA). Blots were blocked for 2 hours at RT in TBS/ 0.1 % Tween/ 5 % skim milk powder (Fluka, Buchs, Switzerland). Blots were subsequently incubated with 2 µg/ml monoclonal antibody specific for secretory component (NI194-4) in TBS/ 0.1 % Tween/ 2.5 % skim milk powder overnight at 4 ºC. After washing with TBS/ 0.1 % Tween, blots were incubated with HRP conjugated goat anti-mouse immunoglobulin (Dako) for 2 hours at RT. After washing bands were visualized with Supersignal (Pierce Chemical Co., Rockford, IL) and exposure to HyperfilmTM films (Amersham Pharmacia).

**Statistical analysis**

Statistical analysis was performed using the Mann-Whitney test. Differences were considered statistically significant when p values are less than 0.05.

## RESULTS

### Specific detection of SIgA in human serum

An ELISA system was developed to specifically measure the amounts of secretory IgA in serum. In this system anti-SC antibodies were coated, samples were applied and SIgA was detected with anti-IgA antibodies. Purified SIgA was readily detected by this ELISA with a detection limit of 100 ng/ml (Figure 1A). In contrast, purified monomeric serum IgA is not recognized in the ELISA, even when applied at high concentrations (Figure 1A). In accordance with previous publications (23), a specific signal for SIgA could be detected in serum of healthy individuals (Figure 1B). The specificity of this assay is especially dependent on the specificity of the anti-SC antibody. Using Western blot, we showed that this monoclonal antibody only recognized the 75 kDa SC (Figure 2A). Furthermore comparison of the NI194-4 antibody with another anti-SC antibody 3F8 (38) showed specificity for SIgA both in a direct ELISA (Figure 2B) as well as in a sandwich ELISA (Figure 2C).

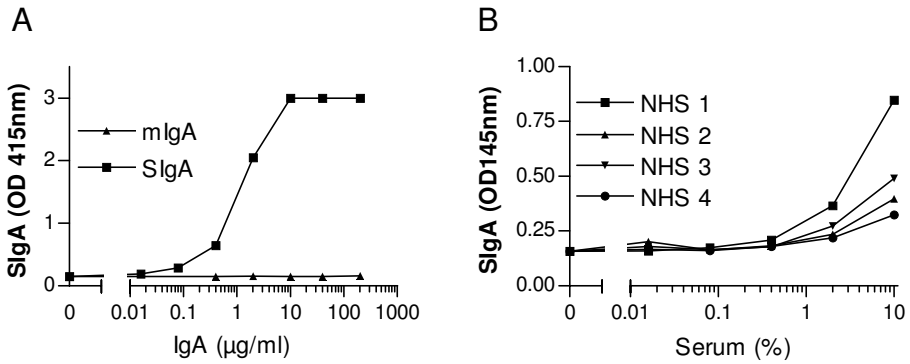


***Figure 1: Specific detection of SIgA in human serum.***
*A) Purified SIgA and monomeric serum IgA were measured in a sandwich ELISA, as detailed in the Material and Methods. B) Measurement of SIgA in serum from healthy individuals in different serum dilutions. NHS, normal human serum.*

To determine the molecular size of SIgA in serum, IgA was isolated from serum using affinity chromatography. Size-fractionation revealed that SIgA was specifically present in the HMW fractions (Figure 3A). Using the same procedure, IgA was isolated from 8 healthy controls followed by gel filtration. Pools containing pIgA and mIgA, respectively, were obtained and assessed for the amount of SIgA (Figure 3B). In all cases, SIgA was demonstrated exclusively in the pIgA pool.

Next we assessed concentrations of SIgA in serum of 47 IgAN patients and 19 healthy controls (Figure 4A). Both in controls and in patients significant serum concentrations of SIgA were detected. There was no significant difference ($p= 0.159$) in the SIgA concentrations in serum of patients (3.3 ± 3.0 µg/ml) compared to controls (2.2 ± 1.2 µg/ml). After comparison the SIgA concentration in serum of IgAN patients with different clinical parameters there were no correlations found. However concentrations of SIgA in IgAN patients were more heterogeneous than in controls. Therefore we divided the IgAN group in patients with elevated levels of SIgA (> 5 µg/ml, mean ± 2 SD of control sera), and patients with normal levels (<5 µg/ml).

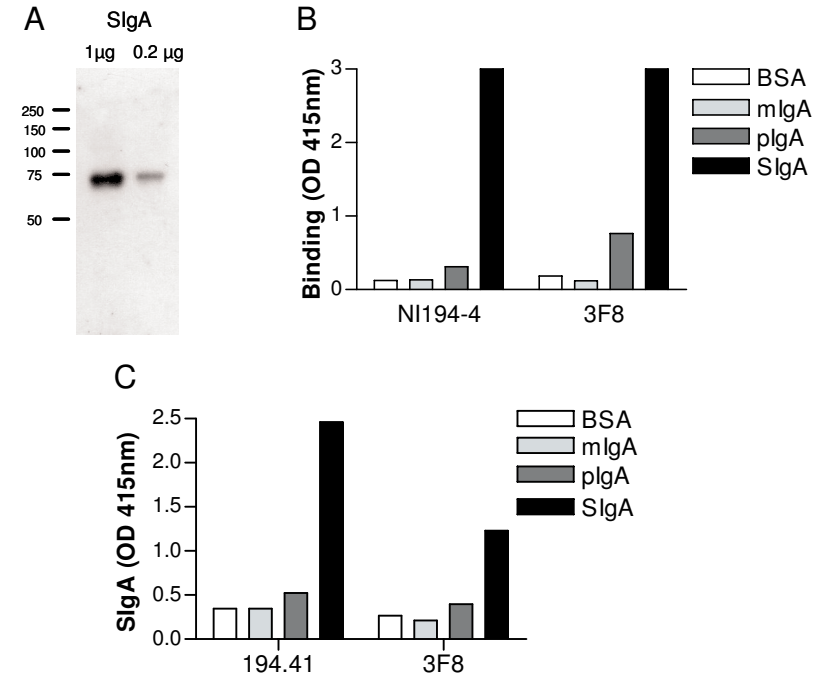


***Figure 2: Specific detection of SIgA and secretory component.***
*A) Detection of secretory component in SIgA, 1 and 0.2 µg SIgA were loaded on 10 % SDS-PAGE gel under reducing conditions after blotting secretory component was detected with 2µg/ml NI194-4. B) Detection of secretory component with NI194-4 and 3F8 (2 µg/ml) in different forms of IgA (2 µg/ml) coated on ninety-six well Nunc maxisorp microtitre plate. C) Different forms of IgA measured with sandwich ELISA using two different coating antibodies (NI194-4 and 3F8).*

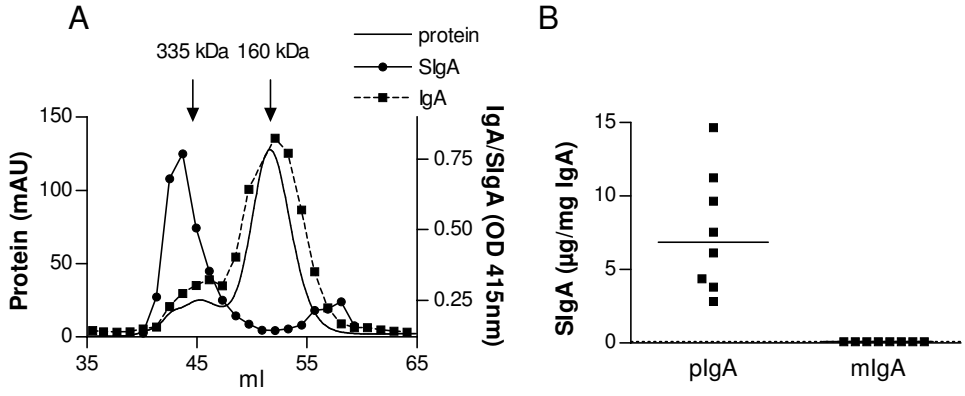


***Figure 3: SIgA is present in high molecular weight fractions of serum IgA.***
*A) IgA was affinity purified and size-fractionated on a HiLoadTM 16/60 HR200 Superdex prep grade gelfiltration column. All fractions were measured for total protein and the presence of total IgA and SIgA by ELISA. B) From 8 healthy controls, IgA was purified and size fractionated as above. IgA was pooled in pIgA fraction (39-47.5ml) and mIgA fraction (47.5-57ml). Both IgA and SIgA concentrations were determined, and depicted is the amount of SIgA corrected for the amount of total IgA The horizontal dashed line represents the detection limit. The horizontal solid lines indicate the median.*

There were no significant correlations with the degree of proteinuria or creatinine clearance and the serum concentration of SIgA (Figure 4B). However, there was significantly more pronounced hematuria in the group with higher levels of SIgA (p= 0.04) (Figure 4B).

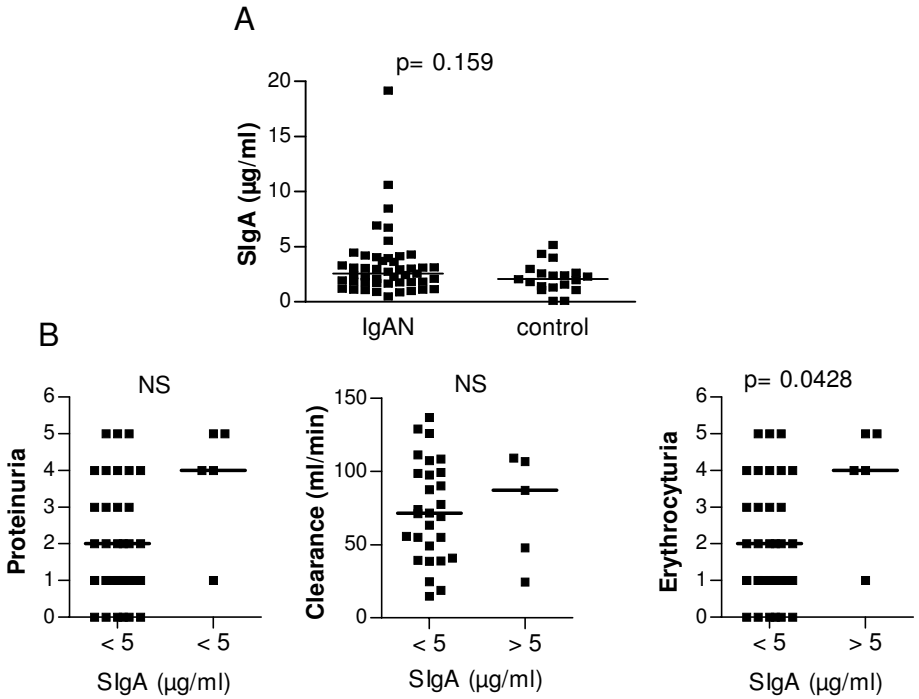


***Figure 4: Measurement of SIgA in serum of IgAN patients and controls and relation to clinical parameters.***

*A) The concentrations of SIgA were determined in serum from patients (n= 47) and controls (n=19). The horizontal dashed line represents the detection limit and the horizontal solid lines indicate the median B) Based on SIgA concentrations IgAN patients were divided in two groups: normal SIgA levels (<5µg/ml; mean ± 2 SD of control sera) and increased SIgA levels (≥ 5µg/ml). These groups were analyzed for proteinuria, creatinin clearance (700* creatinin in urine (mmol/ 24 hours)/ creatinin in serum (µmol/l) and erythrocyturia at the time of sampling. The horizontal solid lines indicate the median. Statistics were performed using the Mann-Whitney test (ns= not significant)*

**SIgA binds to mesangial cells and induces cell activation**

After demonstrating the presence of SIgA in the circulation, we investigated its capacity to interact with mesangial cells. After incubation of the mesangial cell-line AMC11 or normal human mesangial cells (NHMC) with 200 µg/ml of different molecular forms of IgA, the binding was examined by FACS analysis. Binding of monomeric serum IgA to NHMC is very low (Figure 5A). In contrast, the polymeric form of serum IgA showed a clear binding to mesangial cells. However the best binding was observed with similar concentrations of SIgA, which occurred in a dose-dependent fashion, present over a wide range of concentrations (Figure 5B). Although the mean fluorescence intensity of IgA binding to NHMC, compared with

AMC11, was overall higher for all forms of IgA, the relative differences between the different forms of IgA were the same.

The different molecular forms of IgA were compared for their capacity to induce IL-6 production by mesangial cells. Stimulation of NHMC for 72 hours with IgA resulted in an increased IL-6 production. This increase was most prominent following stimulation with SIgA (Figure 6A). The induction of IL-6 production by mesangial cells was dose dependent: a 17-fold increase was observed compared to the negative control upon stimulation with 200 µg/ml SIgA (Figure 6B).

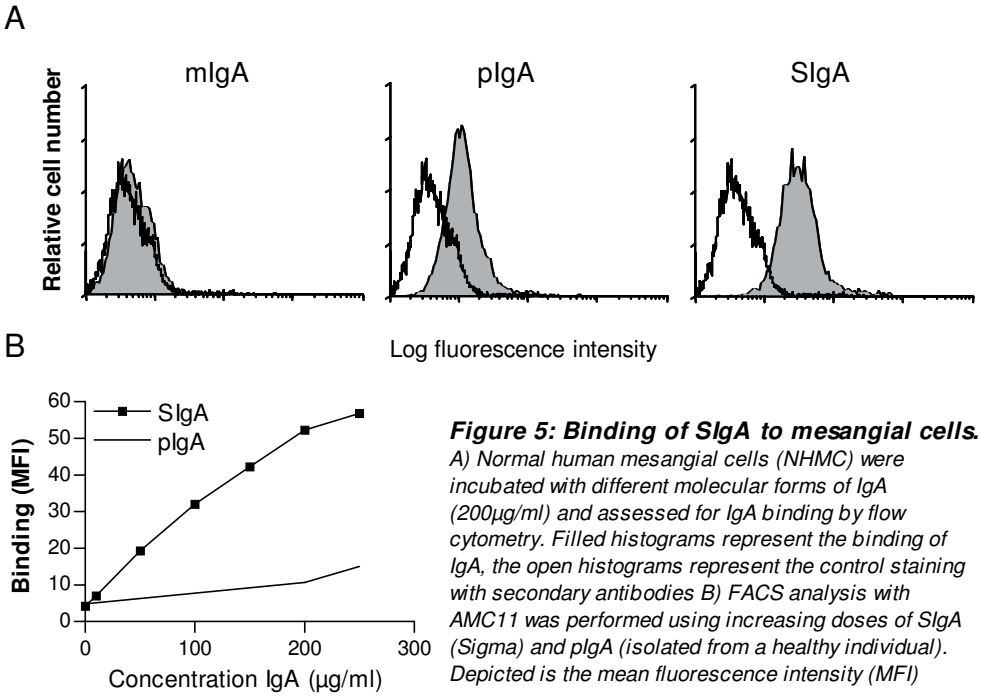


***Figure 5: Binding of SIgA to mesangial cells.*** *A) Normal human mesangial cells (NHMC) were incubated with different molecular forms of IgA (200µg/ml) and assessed for IgA binding by flow cytometry. Filled histograms represent the binding of IgA, the open histograms represent the control staining with secondary antibodies B) FACS analysis with AMC11 was performed using increasing doses of SIgA (Sigma) and pIgA (isolated from a healthy individual). Depicted is the mean fluorescence intensity (MFI)*

**Mesangial cells do not express the mannose receptor (CD206) or MAC-1 (CD11b/CD18)**

Recently we showed that dendritic cells are able to bind SIgA via the mannose receptor (CD206) (40). Therefore we investigated the presence of the mannose receptor as a potential SIgA receptor on mesangium cells. However both using FACS analysis (Figure 7A) and RT-PCR (Figure 7B) we were not able to demonstrate the presence of the mannose receptor on mesangial cells. In both cases, DC served as a positive control. Similarly, we were not able to demonstrate the presence of CD11b/CD18 (Figure 7C), recently identified as a co-receptor for SIgA binding (41).

**Binding of SIgA to mesangial cells is not inhibited by secretory component, EDTA and calcium**

To investigate in more detail the mechanism of binding of SIgA to mesangial

cells, the cells were pre-incubated with free SC. After pre-incubation with SC the binding of SIgA to mesangial cells was not affected (Figure 8A). Binding of SIgA was slightly inhibited with SIgA and pIgA but not with mIgA (Figure 8A), suggesting interaction with the IgA part of the molecule.

To investigate the potential contribution of C-type lectins, a family of cell surface molecules including the mannose receptor, the role of calcium in SIgA binding to human mesangial cells was examined. However, neither the addition of extra calcium, nor the removal of calcium using EDTA showed a significant effect on the binding of SIgA to mesangial cells (Figure 8B).

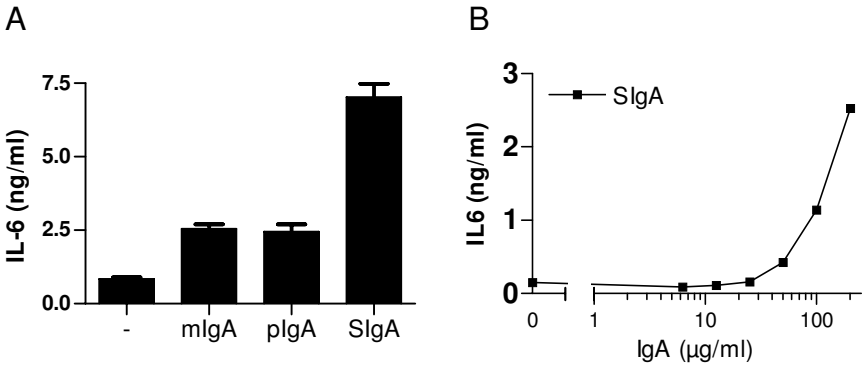


***Figure 6: SIgA increase IL-6 production by mesangial cells.***

*A) Normal human mesangial cells (NHMC) (25x10$^3$ cells/well) were stimulated with different molecular forms of IgA (200µg/ml). After 72 hours supernatants were harvested and tested for IL-6 using ELISA. Depicted is the mean ± SD B) NHMC was stimulated with different concentrations of SIgA (Sigma) as described above and IL-6 production was assessed.*

**SIgA is present in glomerular eluate from a kidney of an IgAN patient.**

To determine the potential role of SIgA in the pathogenesis of IgAN, we had the unique opportunity to analyse the glomerular eluate of a nephrectomized specimen derived from a patient with recurrent IgAN. After elution, concentrations of specific immunoglobulin isotypes were determined. In the glomerular eluate, all immunoglobulin classes measured were detectable (Table 2), including SIgA in a concentration of 2 µg/ml. To exclude that the immunoglobulins in the eluate were the result of aspecific trapping from the circulation, serum immunoglobulin levels were determined in the serum of this patient at the time of nephrectomy (Table 2). The ratio of the immunoglobulin concentrations in the eluate and the serum can be used as a measure of the specific accumulation in the glomerular deposit (Figure 9). In this analysis we observed a ratio for SIgA which was 120 fold higher that the ratio for IgA1.

## DISCUSSION

This is the first study to support a role for SIgA in the pathogenesis of IgAN. We show that SIgA is present in low concentrations in serum of healthy individuals as well as in IgAN patients. In patients with higher SIgA serum concentrations hematuria is more pronounced. Furthermore we show that SIgA exhibits the strongest

binding to mesangial cells compared to serum IgA. Finally, in the eluate of glomeruli from a kidney of an IgAN patient a strong accumulation of SIgA was detected. Taken together these data suggest an important role for SIgA in the pathogenesis of IgAN.

The high incidence of IgAN recurrence after renal transplantation, and the disappearance of deposits of IgA from accidentally transplanted kidneys clearly suggests that intrinsic alterations and/or structural characteristics of IgA contribute to the process of deposition (42,43). The predominance of IgA1 deposits and the specific hinge region of IgA1 with potential O-linked glycosylation sites, has initiated a directed search for alterations in glycosylation. Indeed, both in serum but, more importantly, also in the eluate of renal deposits (14), a specific reduction of O-linked galactosylation has been observed (10,12,14). Furthermore, with size fractionation of eluted proteins from kidney sections, it was shown that deposited IgA was mostly HMW of nature (13). In addition based on different staining methods, it has been proposed that a large part of the deposited IgA is high molecular weight of nature (44-46).

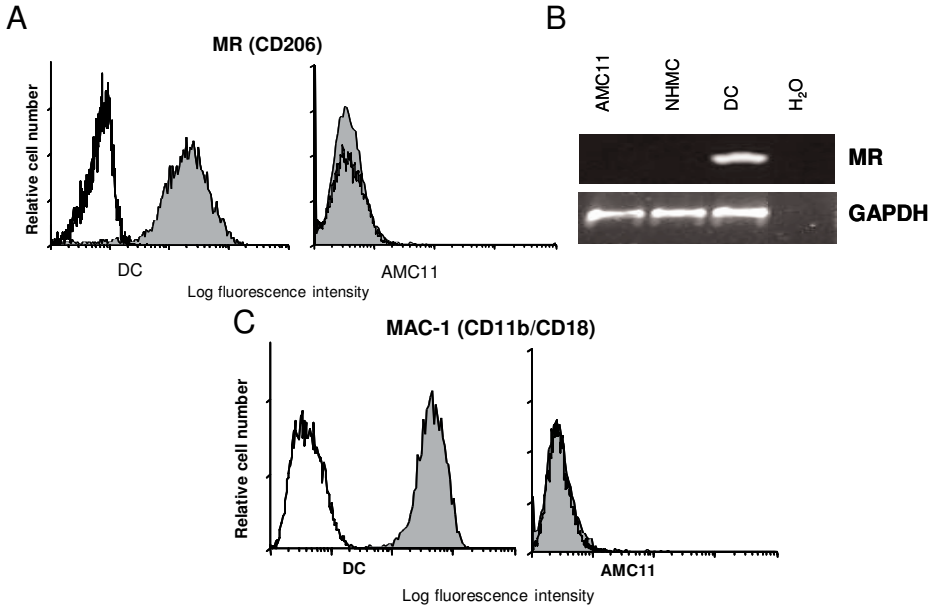


***Figure 7: Mannose receptor and MAC-1 are not present on mesangial cells.***
*A) FACS analysis of mannose receptor on DC and AMC11. Filled histograms represent expression of mannose receptor; open histograms represent the control staining with secondary antibodies B) Mannose receptor mRNA expression was analyzed by RT-PCR as described in the material and methods C) Presence of MAC-1 on mesangial cells and DC was tested with FACS analysis. Filled histograms represent expression of MAC-1. Open histograms represent the control staining with secondary antibodies.*

The results from all these methods provide indirect indications for the composition of the IgA1 deposits. We now show by eluting glomeruli, that glomeruli show a strong and specific accumulation of SIgA compared to other serum immunoglobulins. However, this technique can only be applied in limited cases of situations. We have tried to demonstrate the presence of SIgA deposits using traditional immunofluorescense on cryosections. Until now, we were not able to show SIgA in renal

sections, even not in cryosections of the kidney used for our elution study. This might be due to inappropriate reagents or conformational changes of the deposited SIgA, thereby masking the SC epitope. Therefore it will be necessary to generate other reagents for the detection of deposited SIgA, and a more thorough analysis of renal biopsies.

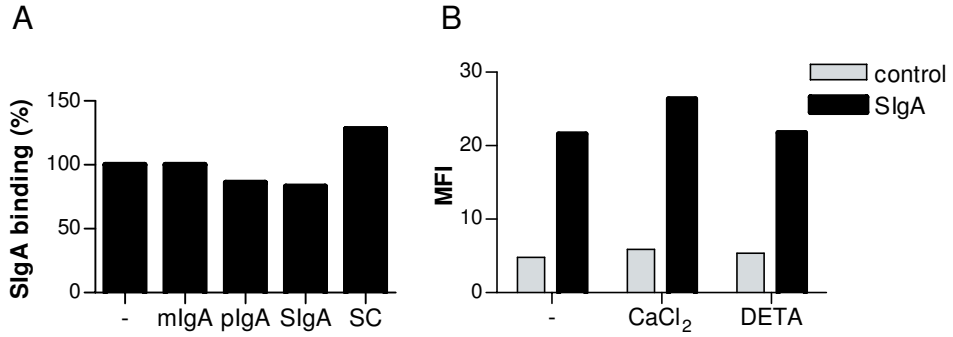


***Figure 8: Binding of SIgA to mesangial cells is not affect by free secretory component or calcium but is affected by IgA.***

*A) Mesangial cells were pre-incubated with mIgA, pIgA, SIgA (400 µg/ml) or free secretory component (SC) (100 µg/ml). After one hour SIgA (200 µg/ml) was added and the binding of SIgA to mesangial cells was examined with flow cytometry. Depicted is the percentage of SIgA binding of a representative experiment of 2 experiments. B) Cells were incubated with SIgA (200 µg/ml) in the presence of absence of EDTA (10 mM) or calcium (10 mM) followed by detection of SIgA binding as described in the Material and Methods.*

As described above, it is assumed that glycosylation of IgA is an important factor in IgAN. Previous studies have shown the role of the glycosylation of IgA on the activation of mesangial cells (47,48), which could be partially explained by altered interaction with mesangial cells (49). The glycosylation of SIgA is different compared to that of serum IgA in several aspects. First, SIgA is a tetra molecular complex consisting of two IgA molecules, a J chain and the SC wrapped around the H chain. Modelling of SIgA suggests that the N-glycans on the heavy chain can be masked by the SC (50). This may also result in a different exposure of the O-glycans. Moreover, specific analysis of the glycosylation of the IgA heavy chain present in SIgA, demonstrated different N-glycan structures compared to that of serum IgA, with terminal GlcNAc residues on the majority of the N-glycans (50). The O-glycans on the hinge region of the heavy chain of SIgA1 presented a wide range of glycan structures, of which the major part is now characterized (50). Finally, also the SC itself is heavily glycosylated. However we were not able to inhibit the binding of SIgA to mesangial cells with SC, suggesting that the SC part is not important for interaction with mesangial cells. It will be a major challenge to isolate SIgA from serum of IgAN patients and to determine specific alterations in glycosylation.

Having shown that SIgA strongly binds to mesangial cells, an important question is which receptor is involved in this binding and whether this receptor is different from that of serum IgA. Several IgA receptors have been described in the literature. The best known receptors, the polymeric Ig receptor (pIgR), the asialoglycoprotein receptor and CD89, have already been described to be absent on mesangial cells (28,51,52). The transferrin receptor is described as an IgA receptor present on

mesangial cells but it has been reported that this receptor is not able to bind SIgA (29). We have previously reported the binding of SIgA to the mannose receptor on dendritic cells (40), but we were not able to demonstrate the presence of the mannose receptor on mesangial cells. Similarly we were not able to demonstrate the presence of CD11b/CD18 (MAC1), a co-receptor for CD89 specifically involved in recognizing SIgA or free SC (41). Therefore until now we have no indication for the mesangial IgA receptor involved in SIgA binding. Our inhibition experiments suggest that IgA rather than SC is recognized and that no C-type lectin is involved. Importantly we found that the putative receptor is able to transmit proinflammatory signals, since SIgA induced a strong dose-dependent increase in IL-6 production by mesangial cells. This seems in contrast with the proposed anti-inflammatory role of SIgA (53).

***Table 2: Immunoglobulin concentrations in serum and glomerular eluate of IgAN patient.***

| | IgA1 | IgA2 | SIgA | IgM | IgG |
|---|---|---|---|---|---|
| Serum (mg/ml) | 5.9 | 0.59 | 0.016 | 0.74 | 10 |
| Glomerular eluate (µg/ml) | 6.1 | 0.65 | 2 | 2.2 | 6.8 |

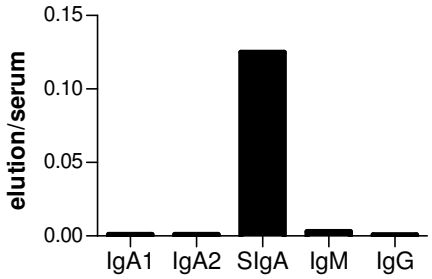


***Figure 9: SIgA is accumulated in the glomerular eluate of an IgAN kidney***
*To make an estimate for the specific accumulation in the glomerular deposit, immunoglobulin concentrations in the eluate and serum were compared. Depicted is the ratio of concentration of different immunoglobulins in the eluate and the serum.*

Generation of SIgA, i.e. production of dIgA followed by transcytosis using the pIgR, is a specific process taking place at mucosal surfaces (54). Interestingly, IgAN patients often present macroscopic hematuria following upper respiratory tract infections. Mucosal challenge also leads to an increased production of IgA in the systemic compartment, probably based on the migration of B cells (the mucosa-bone marrow axis) (55). This mucosa-bone marrow traffic has been confirmed by challenging healthy individuals intranasally with the neoantigen cholera toxin subunit B (CTB) (56). In patients with IgAN we observed a reduced mucosal IgA response to mucosal immunization with CTB (56). At present it is not clear whether a mucosal challenge also regulates levels of circulating SIgA. Still our finding of glomerular accumulation of SIgA provides a link between the mucosal immune system and renal deposits.

In summary, we have shown that SIgA is able to bind to mesangial cells in a

dose-dependent manner and that this binding is calcium-independent and cannot be inhibited with free SC. Furthermore low concentrations of SIgA can be detected in serum. Further research is needed to determine to which receptor SIgA can bind and what the mechanism of cell activation induced by SIgA is. SIgA is strongly accumulated in the glomeruli of a kidney from an IgAN patient. Therefore we conclude that our data support a role for SIgA in the pathogenesis of IgAN, and further research to define such a pathogenic role is warranted.

**Acknowledgements**

The authors would like to thank D.J. van Gijlswijk-Janssen, M.C. Faber-Krol and N. Klar-Mohamad (Dept. of Nephrology, LUMC, The Netherlands) for excellent technical assistance. This work was financially supported by the Dutch Kidney Foundation (BO: C99.1822; AR: PC95)

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