IgA-containing immune complexes in the urine of IgA nephropathy patients

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Abstract

Background. Sera of IgA nephropathy (IgAN) patients contain high levels of circulating immune complexes composed of IgA1 molecules with aberrantly glycosylated hinge-region O-linked oligosaccharides and IgG or IgA1 antibodies with anti-glycan or anti-hinge-region peptide specificities. Due to damaged sieving properties of the glomerular capillary wall in IgAN, these immune complexes may appear in the urine.

Methods. We collected urine samples from 29 patients with biopsy-proven IgAN (Group I), 27 proteinuric patients with non-IgA nephropathies (Group II) and 28 healthy volunteers (Group III). The levels of urinary IgA and IgG and IgA–IgG-containing immune complexes were measured by ELISA and standardized for urinary creatinine concentrations.

Results. The urinary IgA and IgG levels were significantly higher in Groups I and II than in Group III. Although the excretion of IgA as a fraction of total urinary protein was not significantly greater in IgAN patients than in patients with other renal diseases, the excretion of aberrantly glycosylated IgA1 was observed by western blot in 68% of the IgAN patients but in none of the healthy controls. The urinary levels of IgA–IgG-containing immune complexes were measured by ELISA and standardized for urinary creatinine concentrations.

Conclusion. The amounts of urinary IgA–IgG-containing immune complexes were significantly higher in patients with IgAN than in patients with non-IgA nephropathies or healthy controls.

Keywords: ELISA; IgA-containing immune complexes; IgA nephropathy; western blot

Introduction

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis in the world with an estimated incidence of 12–50 patients/million/year. The initial reports described IgAN as a benign glomerulonephritis with microscopic haematuria and modest proteinuria. Currently, it is well recognized that chronic renal insufficiency is a common long-term outcome [1].

The pathogenesis of IgAN is still obscure, but new concepts have emerged during the last decade. Several investigators have shown that a small portion of circulating IgA1 molecules is deficient in galactose (Gal) [2–4]. This abnormality is limited to the O-linked glycans of the hinge region, the unique proline-, threonine- and serine-rich structure between the CH1 and CH2 domains of the IgA1 heavy chain. In the absence of Gal, the terminal sugar N-acetylgalactosamine (GalNAc), sialylated GalNAc or hinge-region glycopeptides are recognized as epitopes by naturally occurring IgG or IgA1 antibodies, and circulating immune complexes are formed [5]. These complexes apparently deposit in the renal mesangium because Gal-deficient IgA1 has also been detected in situ in the glomeruli of IgAN patients [6,7].

Various immunological abnormalities have been described in IgAN patients. About half of them have high levels of serum IgA and nearly as many also show high levels of IgA–C3 and IgA–IgG circulating immune complexes; one-third of the patients have circulating IgA rheumatoid factor [8].
On the other hand, little is known about the composition of the proteins in the urine of IgAN patients. In the early clinical stages, proteinuria is frequently modest. With disease progression, proteinuria usually increases and is often non-selective to include high-molecular-weight proteins (such as immunoglobulins). Indeed, urinary concentrations of IgA and IgG are higher in patients with IgAN than in patients with other renal diseases and healthy controls, and correlate with the magnitude of proteinuria [9]. Due to damaged sieving properties of the glomerular capillaries, proteins of even higher molecular mass, such as circulating immune complexes, may appear in the urine. We investigated the presence and properties of these complexes in urine samples from patients with IgAN and controls.

Subjects and methods

Patients and controls

Serum and urine specimens from IgAN patients (Group I, 29 patients), patients with other renal diseases (Group II, 27 patients) and healthy volunteers (Group III, 28 individuals) were analysed. Serum and urinary creatinine and urinary protein were measured quantitatively in the hospital laboratory. Group I comprised 19 men and 10 women (one patient had recurrent IgAN after kidney transplantation), with mean age 45.3 ± 17.5 years (range, 14–73 years). The mean serum creatinine level was 2.08 ± 1.38 mg/dl and the mean urinary protein/creatinine ratio was 0.13 ± 0.17. Six patients had been treated with glucocorticoids. Group II included 13 men and 14 women, with mean age 58.7 ± 14.1 years (range, 22–82 years). The diagnoses included non-IgA mesangial glomerulonephritis (n = 5), lupus nephritis (n = 2), chronic pyelonephritis or interstitial nephritis (n = 5), diabetic nephropathy (n = 7) and renal engraftment (non-IgA glomerulonephritis, n = 4; interstitial nephritis, n = 3; Alport’s syndrome, n = 1). Twelve had been on glucocorticoid treatment for various intervals. The mean serum creatinine level in this group was 2.94 ± 1.80 mg/dl, and the mean urinary protein/creatinine ratio was 0.29 ± 0.40. Hypertensive patients in Groups I and II were treated preferentially with ACE inhibitors and/or angiotensin II type 1 receptor blockers. Group III consisted of 10 men and 18 women with mean age 46.6 ± 13.8 years (range, 17–63 years). These individuals had normal blood pressures and serum creatinine levels (0.84 ± 0.16 mg/dl) but no proteinuria by dip stick.

The study was approved by the Institutional Review Board at the University of Alabama, Birmingham; informed, written consent was obtained before collecting samples.

Determination of IgA and IgG concentrations in urine and serum

IgA and IgG concentrations and levels of IgA–IgG-containing immune complexes in urine and serum were determined by enzyme-linked immuno sorbent assay (ELISA). Corresponding myeloma proteins were used as standards. Wells of microtitre plates were coated with 1 μg/ml F(ab')2 fragment of goat anti-human IgA (for cross-capture of IgA–IgG complexes) or 1 μg/ml F(ab')2 goat anti-human IgG (for cross-capture of IgG–IgA complexes) (both capture antibodies from Southern Biotechnology Associates, Birmingham, AL, USA). After washing and blocking with bovine serum albumin (BSA; Sigma Chemical Company, St Louis, MO, USA) in phosphate buffered saline (PBS, pH 7.3) containing 0.05% Tween-20 (BSA-PBS-Tween), the samples were diluted 11-fold with the same buffer. After 2 h incubation at 37°C in a humidified chamber, the test samples were removed, wells were washed with BSA-PBS-Tween and incubated for 2 h at 37°C with biotin-labelled goat F(ab')2 anti-human IgG (BioSource International, Camarillo, TX, USA) diluted 1:10 000 (termed ‘IgA–IgG complexes’) or biotin-labelled F(ab')2 anti-human IgA (BioSource) diluted 1:10 000 (termed ‘IgG–IgA complexes’). After washing the plate with the same buffer, the wells were incubated with avidin-alkaline phosphatase diluted 1:8000 (Sigma) for 2 h in a humidified chamber. After additional washings, the wells were incubated with the phosphatase substrate (Sigma) and the optical density (OD) was read at 405 nm using an EL 808 Ultra Microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Determination of Gal-deficient IgA1 by ELISA

High-adsorption polystyrene 96-microwell plates (Nalge Nunc International, Rochester, NY, USA) were coated overnight with 3 μg/ml F(ab')2; fragment of goat IgG anti-human IgA (Jackson Immunoresearch Labs, West Grove, PA, USA) in PBS. Coated plates were blocked with 1% BSA (Sigma) in PBS 0.05% Tween-20 (PBST) and serial 2-fold dilutions of duplicate samples and standards in blocking solution were incubated overnight at room temperature. Gal-deficient IgA1 isolated from the serum of an IgA1 myeloma patient was used as standard [5]. The bound IgA was detected by incubation with biotin-labelled F(ab')2; fragment of goat IgG anti-human IgA (BioSource) for 3 h at 37°C followed by 1 h incubation with horseradish peroxidase (HRP)-conjugated ExtrAvidin (Sigma). For better detection of Gal-deficient IgA1, the terminal sialic acid was removed by incubation for 3 h at 37°C with 10 μM neuraminidase (Roche Diagnostic Corp., Indianapolis, IN, USA) in 0.01 M acetate buffer pH 5 [5]. After washing, 100 μl biotin-labelled GalNAc-specific lectin isolated from Helix aspersa (HAA; Sigma; 1:500 dilution) was added to each well. Following 2 h incubation at 37°C, the plates were washed with HRP-ExtrAvidin (Sigma) and incubated for another hour at 37°C. The wells were developed with the peroxidase chromogenic substrate O-phenylenediamine (OPD)-H2O2 (Sigma). The colour reaction was stopped with 1 M sulphuric acid, and the absorbance at 490 nm was measured using an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments). The amount of Gal-deficient IgA1 in the tested samples was calculated by interpolating the OD values on calibration curves, constructed using Gal-deficient IgA1 myeloma protein with the DeltaSoft II program (BioMetallics, Inc., Princeton, NJ, USA).

Estimation of molecular mass of urine and serum proteins

To determine the molecular mass of IgA-containing immune complexes in the urine and serum, 0.5 ml urine or 0.5 ml
serum from selected patients and controls was filtered through a 0.45 µm filter (Pall Corporation, Ann Arbor, MI, USA) and fractionated on a calibrated Superose 6 column (600×12 mm) equilibrated in PBS. The microtitre plates coated with goat F(ab)2 anti-human IgA or goat F(ab)2 anti-human IgG were incubated with individual column fractions and developed with biotin-labelled monoclonal anti-human IgA1 (diluted 1:2000), clone 69-11.4 or goat F(ab)2 anti-human IgG, as described earlier.

Western blot
Proteins from urine fractionated by a gradient (4-20%) polyacrylamide gel electrophoresis (Bio-Rad laboratories, San Francisco, CA, USA) with SDS and β-mercaptoethanol were blotted for 1 h on Immobilon P (Millipore Corporation, Bedford, MA, USA) and stained with the following: (i) Coomassie Blue to detect proteins; (ii) biotinylated goat F(ab)2 fragment against human IgA (BioSource) to detect α-immunoglobulin heavy chain; and (iii) biotinylated HAA to detect glycoproteins with terminal GalNAc residues. Interactions of IgA1 with human IgG anti-IgA1 antibodies were assayed by incubation of western blots with biotin-labelled IgG from IgAN patients. The samples for lectin studies were de-sialylated at pH 6 with neuraminidase from Vibrio cholerae (Roche, Mannheim, Germany). The detection of biotinylated IgG, IgA and HAA was conducted by chemiluminescence using Supersignal (Pierce Chemical Company, Rockford, IL, USA) and Biomax film for chemiluminescence (Amersham, Biosciences Corp., Piscataway, NJ, USA).

Statistical analysis
Levels of urinary IgA, IgG and IgA-containing immune complexes were standardized to the urinary creatinine concentration to adjust for differences in daily urinary volume [10]. Original data were log-transformed and analysis of covariance was used to compare the groups. Proteinuria was selected as a covariate to diminish its influence on other variables. The analyses were performed with SYSTAT® statistical software (SYSTAT Software Inc., Richmond, CA, USA). All tests were two-tailed and P < 0.05 was considered significant.

Results

Urinary IgA and IgG concentrations
The urinary IgA and IgG concentrations (adjusted for urinary creatinine concentration) varied widely in IgAN patients (Group I) and patients with other renal diseases (Group II) (Table 1). Their values were significantly higher than in healthy controls (Group III). The levels of urinary IgA and IgG correlated in all three Groups (R = 0.6799, 0.7283 and 0.7605 for Groups I, II and III, respectively; P < 0.001 for all groups). The urinary excretion of IgA correlated with proteinuria (R = 0.6799 and 0.7605 in Groups I and II; P < 0.001 for both groups), but did not correlate with the serum creatinine concentration.

When the urinary excretion of IgA was expressed as a fraction of total urinary protein (calculated as urinary IgA concentration divided by urinary protein concentration), IgA constituted a higher (although not statistically significant) fraction of total proteinuria in Group I than in Group II (median value 0.62 for Group I and 0.23 for Group II). The corresponding median values for IgG were 0.80 and 0.45 for Group I and Group II, respectively, and did not differ significantly.

We also calculated the total 24 h urinary IgA and IgG excretion in the healthy subjects (Group III) using an estimated urinary creatinine excretion of 21 mg/kg/24 h for males and 19 mg/kg/24 h for females [10]. The values for IgA ranged from 0.5 µg/kg/24 h to 9.1 µg/kg/24 h (median 3.2). The values for IgG ranged from 0.5 to 42.8 µg/kg/24 h (median 10.0). The excretion of IgA and IgG for patients in Groups I and II was about 10-fold higher and directly correlated with proteinuria.

Levels of IgA-containing immune complexes in the urine
The levels of urinary IgG–IgA immune complexes and IgA–IgG immune complexes in Group I were significantly higher than those in Groups II and III (Tables 2 and 3). Group II did not significantly differ from Group III. A highly significant correlation was found between the urinary levels of both types of complexes in all three groups (R = 0.8193, P < 0.001 for the three groups combined; R = 0.7370, P < 0.001; R = 0.7752, P < 0.001; and R = 0.7652, P < 0.001 for Groups I, II and III, respectively). The levels of IgA-containing complexes correlated with magnitude of proteinuria (P < 0.01) in Groups I and II, but not with serum creatinine concentration.

Because glucocorticoids may influence the glomerular capillary permeability for macromolecules,
Urinary IgA-containing immune complexes in IgAN

**Table 2. Urinary IgG–IgA complexes**

<table>
<thead>
<tr>
<th>Group Ib</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>0.4945</td>
<td>0.2633</td>
</tr>
<tr>
<td>SD</td>
<td>0.4237</td>
<td>0.3690</td>
</tr>
<tr>
<td>Median</td>
<td>0.3259</td>
<td>0.1236</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.0010</td>
<td>0.0050</td>
</tr>
<tr>
<td>Maximum</td>
<td>1.6230</td>
<td>1.2980</td>
</tr>
</tbody>
</table>

*aUrinary IgG–IgA complexes (plates coated with anti-IgG antibody for capture and developed with anti-IgA antibody).*

Statistical significance (calculated using average±SD values):

- Group I vs Group II, P < 0.01
- Group I vs Group III, P < 0.05
- Group II vs Group III, NS.

**Table 3. Urinary IgA–IgG immune complexes**

<table>
<thead>
<tr>
<th>Group Ib</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>0.4795</td>
<td>0.2810</td>
</tr>
<tr>
<td>SD</td>
<td>0.2845</td>
<td>0.2526</td>
</tr>
<tr>
<td>Median</td>
<td>0.4455</td>
<td>0.1820</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.0225</td>
<td>0.0320</td>
</tr>
<tr>
<td>Maximum</td>
<td>1.1410</td>
<td>0.8455</td>
</tr>
</tbody>
</table>

*aUrinary IgA–IgG complexes (plates coated with anti-IgA antibody for capture and developed with anti-IgG antibody).*

Statistical significance (calculated using average±SD values):

- Group I vs Group II, P < 0.01
- Group I vs Group III, P < 0.05
- Group II vs Group III, NS.

we also analysed the data after exclusion of the treated patients. The levels (average±SD) of both types of IgA-containing complexes remained about 2-fold higher in Group I than in Group II (P < 0.05).

**Urinary IgA-containing immune complexes as a component of total urinary protein excretion**

To determine the relative contribution of IgA-containing complexes to the total urinary protein excretion, we calculated an index: IgG–IgA (or IgA–IgG) urinary immune complexes concentration divided by urinary protein concentration (mg/dl) and multiplied by 100. Statistical significance (calculated using average±SD values): Group I vs Group II, P < 0.01; Group I vs Group III, P < 0.05; Group II vs Group III, NS.

**Table 4. The contribution of IgA-containing immune complexes to total urinary protein**

<table>
<thead>
<tr>
<th>Group Ib</th>
<th>Group II</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG–IgA complexes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.76</td>
<td>0.09</td>
<td>0.72</td>
</tr>
<tr>
<td>SD</td>
<td>0.93</td>
<td>0.05</td>
<td>0.86</td>
</tr>
<tr>
<td>Median</td>
<td>0.58</td>
<td>0.07</td>
<td>0.52</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Maximum</td>
<td>4.26</td>
<td>0.17</td>
<td>3.84</td>
</tr>
</tbody>
</table>

*aUrinary IgG–IgA complexes (plates coated with anti-IgG antibody for capture and developed with anti-IgA antibody).*

Statistical significance (calculated using average±SD values):

- Group I vs Group II, P < 0.01 for IgG–IgA immune complexes; P < 0.001 for IgA–IgG complexes.

Immune complexes were found in fractions with a corresponding molecular mass ~650–900 kDa. The IgG–IgA complexes in serum fractions were identified in a more narrow range of molecular mass with the peak at 750–900 kDa. In contrast to IgAN patients, disease controls had very low levels of high-molecular-mass IgA–IgG complexes in the urinary fractions (Figure 1B). These results are in agreement with the ELISA data (Table 4).

**Comparison of serum and urinary IgA-containing immune complexes**

To determine the levels of IgA-containing immune complexes, urine and serum samples of 10 randomly selected IgAN patients and 10 healthy controls were analysed by ELISA. Significantly higher levels of IgA–IgG complexes were found in the sera and urine specimens of IgAN patients than in those of the controls (Figure 2). IgAN patients had high concentrations of urinary IgA–IgG complexes compared with only trace amounts in the normal controls (P < 0.001). We did not find a significant correlation between the concentrations of urinary and serum IgA–IgG complexes in IgAN patients (R = −0.3216).

**IgA-capture lectin ELISA**

Urinary IgA1 showed enhanced reactivity with GalNAc-specific lectin HAA, indicating Gal-deficiency of O-linked glycans. The urine samples from IgAN patients tended to have higher levels of Gal-deficient IgA1 (77.3 ± 99.5 µg/ml) as compared with healthy controls (28.3 ± 38.7 µg/ml) and patients with non-IgAN glomerulonephritis (41.3 ± 27.3 µg/ml), but these values did not reach statistical significance.
Western blot analysis of urinary proteins

Western blot analysis with anti-IgA antibody showed that urine specimens from IgAN patients and patients with other renal diseases contained highly variable amounts of IgA (Figure 3). When developed with biotinylated HAA, the \( \alpha \)-chain of urinary IgA of 13 of 19 IgAN patients (e.g. Figure 3, lanes 6, 7 and 8), none of 13 normal controls and only one of three disease controls displayed binding to the lectin. In addition to the \( \alpha \)-chain, other HAA-binding glycoproteins were detected in the urine of some individuals (e.g. Figure 3, lanes 2 and 6). These urinary proteins, which contain O-linked glycans (e.g. Tamm–Horsfall protein) and
thus can react with HAA, were present in only some IgAN patients and in controls at various levels [11].
Furthermore, IgG with anti-IgA1 specificity isolated from the serum of an IgAN patient also bound α-chains from other IgAN patients (Figure 3). Interestingly, there were some differences with respect to the intensity of α-chain bands developed with anti-α-chain antibody, HAA and IgG. For example, the urine specimen from an IgAN patient (lane 9) displayed bands of a high intensity after development with anti-α chain antibody and IgG but no binding to HAA. On the other hand, α-chains from another patient (lane 8) bound to anti-α-chain antibody and HAA but not to IgG. These results suggest that urinary α-chains display marked heterogeneity of structure or composition of their O-linked glycans.

Discussion

Urinary IgA and IgG concentrations vary widely and are generally higher in patients with IgAN than in patients with other renal diseases [9]. IgA and IgG urinary levels correlate with serum creatinine and the magnitude of proteinuria [9,12,13]. In contrast to healthy individuals, urinary IgA in IgAN patients is predominantly monomeric [9]. In these studies, the urinary excretion of IgA and IgG was reported as concentrations, but not standardized for 24 h urine volume. In the current study, the urinary concentrations of IgA and IgG, standardized for urinary creatinine, correlated with the magnitude of proteinuria. The excretion of IgA as a fraction of total urinary protein was not significantly greater in IgAN patients than in patients with other renal diseases. Thus, our findings did not confirm an earlier observation in children [13]. However, our western blot studies with HAA lectin binding showed an appreciable amount of aberrantly O-glycosylated IgA1 in the urine of IgAN patients. This finding may indicate that measurement of a nephritogenic form of the immunoglobulin in the urine may be of greater diagnostic significance than measurement of total urinary IgA.

The generation and metabolism of IgA-containing immune complexes is a dynamic process [14,15]. These complexes in the circulation consist of IgA1 with Gal-deficient hinge-region glycans and anti-glycan antibody [3,5] of the IgG or IgA1 isotype. IgA in the renal mesangium is likely derived from the deposition of these circulating immune complexes [4,6,14]. Their blood levels are frequently increased in patients with IgAN, especially during a clinical flare-up [4,8,15,16]. These levels correlate with clinical activity of the disease and pathology findings in the renal biopsy specimens [17]. The disappearance of IgA from the mesangium of autologous kidneys has been only rarely documented, even during a clinical remission. Conversely, IgA deposits in a renal allograft from a donor with subclinical IgAN vanished within several weeks after transplantation [18]. These observations indicate that kidneys of IgAN patients are exposed to a high load of circulating IgA-containing immune complexes that exceeds the clearing capacity. Presumably, immune complexes deposit in the mesangium or cross the filtration barrier and thus enter the urine. Indeed, we found significantly higher urinary concentrations of IgA-containing immune complexes in IgAN patients than in patients with other renal diseases or healthy controls. These levels closely correlated with the magnitude of proteinuria. Furthermore, the ratio of urinary IgA-containing complexes to urinary protein was higher in IgAN patients than in patients with other renal diseases.

We did not find a correlation between the serum and urinary concentrations of IgA-containing immune complexes. We postulate that not only ultrafiltration but also other mechanisms may affect the urinary excretion of these complexes in IgAN. For example, the filtration of proteins across the glomerulus is charge-dependent [19]. The IgA1 molecules in the circulating immune complexes of IgAN patients are aberrantly glycosylated [2,4,5]. Thus, this structural abnormality resulting in the immune complex formation may affect the net isoelectric charge and the resultant excretion [20].

Another factor influencing retention of IgA-containing immune complexes in the mesangium is receptor-mediated binding to mesangial cells [14,21,22]. Immune complexes containing Gal-deficient IgA1 bind to mesangial cells more efficiently than uncomplexed IgA1. Some observers have shown that transferrin receptor, CD71, mediates this binding to mesangial cells [21].

In summary, we found higher urinary levels of IgA-containing immune complexes in IgAN patients than in patients with other renal diseases (with a comparable or greater proteinuria) or in healthy individuals. This finding expands the spectrum of abnormal IgA1 metabolism in IgAN [1,4,22].

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Conflict of interest statement. None declared.

References


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