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Antibody response to IgA-binding streptococcal M proteins in children with IgA nephropathy

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Background: IgA nephropathy (IgAN), the most common glomerulonephritis worldwide, is characterized by mesangial deposits containing predominantly IgA. IgAN commonly occurs or exacerbates after upper respiratory tract infections such as streptococcal pharyngitis. Certain group A streptococci express M proteins with IgA-binding regions (IgA-BRs). We have previously shown that these IgA-BRs co-localize with mesangial IgA in IgAN.

Methods: Blood samples from patients with IgAN (n=21) and age-matched controls (n=83) were assayed by ELISA to detect an IgG antibody response to the IgA-BRs of the M4, M22 and M60 proteins. Antibodies were assayed for each IgA-BR separately and the results were combined.

Results: Antibody levels to the IgA-BRs were significantly higher in IgAN patients than controls (p=0.016), particularly in patients with recent streptococcal infection (p=0.008).

Conclusion: The results suggest that children with IgAN had a previous infection with a streptococcal strain expressing an IgA-binding M protein.

**Keywords:** Children, Group A streptococci, IgA nephropathy, M protein
Introduction

IgA nephropathy (IgAN) is a common form of glomerulonephritis characterized by recurrent episodes of macroscopic hematuria, variably progressing to renal failure, in which renal biopsies demonstrate predominantly galactose-deficient IgA1 deposits in the mesangium [1-3]. IgAN is often preceded by infections[4-10], primarily of the upper respiratory tract. Many cases are preceded by streptococcal pharyngitis [10] and clinical observations indicate that tonsillectomy may improve the outcome of IgAN [11].

Group A streptococci (GAS) express surface-localized M proteins, which have an N-terminal hypervariable region that determines the M type of the strain. Certain M proteins bind human IgA-Fc via a semi-variable region (IgA-binding region, IgA-BR) [12-15] . In a previous study we showed that most renal biopsies from pediatric IgAN patients contained mesangial deposits of IgA-BRs, which co-localized with IgA, suggesting a pathogenic role for IgA-BRs in IgAN [6]. The aim of the current study was to analyze the antibody response to IgA-BRs in children with IgAN.
Subjects and Methods

Subjects and blood samples

Patients with IgAN (n=21) and age-matched controls (n=83) were included in the study. Blood (serum or citrated plasma) samples were taken within a median of 3 months (range 0 – 14 months) after the onset of clinical disease (as defined by the presence of symptoms) in all patients. Detailed information, regarding the patients and controls, and blood samples taken, are available as Supplementary data. The study was approved by the ethics committee of the Medical Faculty, Lund University and blood samples were obtained with informed written consent of all patients, or their parents when patients were younger than 15 years.

Evidence for streptococcal infection in patients

Throat cultures and serological assays for streptococcal infection were available for 18 of 21 patients. Evidence for recent streptococcal infection was found in 10 of 18 patients at the initial presentation. For methodology, see Supplementary data.

Synthetic peptides and rabbit antisera

The IgA-BRs of the M4, M22 and M60 proteins were available as synthetic peptides designated Sap4, Sap22 and Sap60, respectively [6]. The N-terminal hypervariable regions of M4 (M4-N) and of the non-IgA binding M5 protein (M5-N) were also available as synthetic peptides [16]. Rabbit antisera to the peptides were raised as described [6, 16].
Detection of IgG antibodies to the IgA-BRs of M4, M22 and M60

Serum IgG antibody levels to the Sap4, Sap22 and Sap60 peptides, and also to the non-IgA-binding M4-N and M5-N peptides, were measured by ELISA. Immunoblotting was used to test the specificity of the secondary antibody. For detailed descriptions, see Supplementary data.

Statistics

Statistical evaluation was performed using SPSS version 17.0 (Chicago, Il). Differences in antibody levels were evaluated by the Mann-Whitney U test. Correlation of antibodies was evaluated using Spearman’s rho test. P values \( \leq 0.05 \) were considered significant.
Results

Serum antibody response to the IgA-BRs of M4, M22 and M60

For all patients and age-matched controls serum antibody levels to the IgA-BRs of the M4, M22 and M60 proteins were assayed for each protein separately and the results were combined (Figure 1). The non-IgA-binding M5-N peptide, from the M5 protein, served as a control. Antibody levels to the IgA-BRs of the M4, M22 and M60 proteins were significantly higher in patients than in controls (p=0.016). This difference became even more significant when the ten sera of patients with evidence for recent GAS infection were compared with controls (p=0.008). Patients with evidence for recent GAS infection had higher levels of antibodies to IgA-BR than patients without (p=0.03).

Patient antibody levels to the IgA-BR of M4 correlated significantly with those to M4-N, derived from the most N-terminal region of M4 (r=0.683, p=0.001), suggesting an antibody response to a larger region of M4.
**Discussion**

In a previous study we presented evidence that patients with IgAN have mesangial deposits of IgA in complex with a streptococcal M protein fragment that binds IgA-Fc, indicating that infection with an IgA-Fc-binding GAS strain contributes to the pathogenesis of IgAN [6]. The present study provides further support for this notion, because patients with IgAN had significantly higher levels of IgG antibodies to streptococcal IgA-BRs. Although IgA-BRs vary in sequence among strains [15], sequence homology may cause cross-reactivity between different IgA-BRs, as described for M4, M22, and M60 [6]. Such cross-reactivity may have permitted demonstration of increased antibody levels also in patients infected by a GAS serotype not included in our assay. Interestingly, analysis of the M4 system suggested that IgAN patients also have antibodies to the most N-terminal region of M4, suggesting an immunological response to a larger part of, or the entire M protein, in these patients. Together, these data provide evidence for a general prevalence of infections, caused by IgA-binding GAS strains, in children with IgAN.
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Transparency declaration

The authors have no conflicts of interest to declare.

The results presented in this paper have not been published previously in whole or part, except in abstract format.
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Absorbance

IgAN
n=21

Controls
n=83

IgAN
GAS
n=10

IgAN
GAS
n=8

IgAN
n=21

Controls
n=83

IgA-BR

M5-N
Figure legend

Figure 1: Antibody levels to the IgA-binding region of the M4, M22 and M60 proteins and the M5-N peptide in IgAN patients and controls. Antibody levels for each individual patient or control resulted in three separate observations, representing antibodies to the IgA-BRs of M4, M22 and M60, that were combined for comparison. Thus, results for IgAN patients (n=21) represent a combination of 63 observations, and results for controls (n=83) represent 249 observations. Levels are displayed as absorbance values obtained by ELISA. The line inside each box represents the median. The upper and lower limits of the box-plot represent the interquartile range. The lower and upper limits represent the range. Higher antibody responses to the three IgA-BRs were found in IgAN patients than controls, especially if the subgroup of patients with evidence for recent GAS infection was compared with controls. No difference between patients and controls was found in antibody levels to the non-IgA binding M5-N.
Supplementary data

Subjects
Patients with IgA nephropathy (IgAN, n=21, 6 girls and 15 boys, median age 13 years, range 3-19) treated at the Department of Pediatrics, Lund University, between 1994 and 2010 were included in the study. IgAN was clinically defined by the presence of glomerulonephritis with hematuria and/or proteinuria. All patients had proteinuria and all underwent renal biopsy showing mesangioproliferative glomerulonephritis with mesangial deposits of IgA. Thirteen of the 21 patients have been previously described [1]. Two patients were treated with steroids before blood samples were taken. No other immunosuppressive treatments had been given.

Control serum samples (n=83, 38 girls, 45 boys, median age 12 years, range 7-18 years) were available from pediatric outpatients suffering from diseases other than IgAN (asthma, growth retardation, diabetes mellitus type 1, status-post pyelonephritis, thrombotic thrombocytopenic purpura, neuroblastoma, neurological disease, opiate intoxication and children investigated for celiac disease in whom this diagnosis was ruled out).
Blood samples

Venous blood from patients and controls was collected in 4 mL vacutainer Hemogard SST tubes for serum or in 5 mL vacutainer tubes, containing 0.5 mL 0.129 mol/L sodium citrate for plasma (tubes from Becton Dickinson, Plymouth, UK). Samples were centrifuged at 2000 g for 10 min and frozen at −20°C until assayed.

Evidence for streptococcal infection in patients

Evidence for GAS infection was investigated by a microbiological method (throat culture at time of onset of IgAN) or serological methods (anti-streptolysin (ASO) and/or anti-DNaseB) assayed at routine hospital laboratories using kits from BioSystem S.A., Barcelona, Spain (ASO) and Dade Behring, Deerfield, IL (anti-DNaseB).

Serum samples were obtained and analyzed within 0-17 months, median 1.5 months, after clinical debut of IgAN. Seven patients with IgAN had a positive serologic assay and three had a positive throat culture for GAS.

ELISA for detection of IgG antibodies to the IgA-BR of M proteins serotype 4, 22, 60 and M4-N and M5-N

Microtiter wells (Maxisorp, Nunc, Roskilde, Denmark) were coated with 150 µl of Sap4, Sap22, Sap60, M4-N or M5-N peptides in solution, all at 2 µg/ml in 0.1 M NaHCO₃ pH 9.6 (Merck, Darmstadt, Germany), and incubated overnight at 4°C. Wells were washed three times with 175 µl PBS-Tween 0.05% (PBS-T, Medicago, Uppsala, Sweden) and unspecific binding-sites blocked with 3% bovine serum albumin (BSA, MP Biomedicals, Irvine, CA) in PBS-T at rt for 2 h, followed by washing and incubation with 100 µl serum or plasma (1:100 v/v) in 0.1% BSA-PBS-T for 1 h at 37°C. After washing, the secondary antibody (rabbit anti human-IgG:horse-radish-peroxidase (HRP, 100µl, Dako, Glostrup, Denmark, 1:2000 (v/v) in
0.1% BSA-PBS-T) was added and the wells were incubated for 1 h at 37°C. Wells were washed and the detection substrate O-phenylenediamine dihydrochloride tablets (Dako) suspended in dH₂O (3 ml/tablet) and 30% H₂O₂ (1.25 µl/tablet) was added for 6 min. The reaction was terminated by addition of 100 µl 1 M H₂SO₄ (Scharlau Chemie, Barcelona, Spain). Absorption was measured at OD₄₉₀ nm.

Plate coating with the appropriate peptide was controlled by ELISA as described above, but after the blocking step wells were incubated with rabbit sera anti-Sap or anti-N terminal peptide against the corresponding serotype 1/100 (v/v) in 0.1% BSA-PBS-T. As the secondary antibody goat-anti-rabbit IgG:HRP (Dako) 1/2000 (v/v) in 0.1% BSA-PBS-T was used.

Sap peptides could be expected to bind IgA in serum/plasma samples, resulting in reduced binding of IgG antibodies due to steric interference. The concentration of Sap used to coat the ELISA wells was therefore determined so that negligible IgA binding occurred. This was tested using microtiter wells coated with 150 µl Sap4, Sap22, or Sap60 in a dilution series from 0.5 to 15 µg/ml. After washing and blocking (as above) wells were incubated with 100 µl normal IgA (from human colostrum 50 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C. After washing, IgA-binding was measured using 100 µl rabbit anti-human-IgA:HRP 1:2,000 (v/v, Dako) in 0.1 % BSA-PBS-T for 1 h at 37°C and detected as above. At a Sap concentration of 2 µg/ml, or less, the Sap peptides exhibited minimal IgA binding in this assay, but retained specific antigenicity for the corresponding rabbit anti-Sap antibodies as described above.
The specificity of IgG detection was demonstrated by IgG depletion of sera by passage through a Protein G-sepharose column (Amersham Biosciences, Uppsala, Sweden).

**Immunoblotting to test the specificity of the secondary antibody**

The IgG specificity of the secondary antibody (rabbit-anti-human-IgG:HRP) was tested by immunoblotting. Briefly, sera from two controls 1/50 (v/v) and IgG-depleted sera from the same controls 4/50 (v/v) in reducing sample buffer (4% (w/v) sodium dodecyl sulfate (SDS), Bio Rad, Hercules, CA, USA) in Tris buffer, pH 6.8 (ICN Biomedicals, Aurora, OH, USA) containing 10% (v/v) 2-mercaptoethanol (Kebo Lab, Spånga, Sweden) were separated by 10% SDS- polyacrylamide gel electrophoresis. The proteins were transferred onto a Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) using a semi-dry electroblotter. The membranes were blocked with PBS-T containing 5% (w/v) dry milk powder (Semper, Sundbyberg, Sweden) and antibodies detected by rabbit anti-human IgG-HRP 1:2000 (v/v) or rabbit anti-human IgA-HRP 1:2000 (v/v) (both Dako) in blocking buffer. Labelling was detected by chemiluminiscence (Amersham ECL, GE Healthcare, Buckinghamshire, UK). Using anti-human-IgG:HRP IgG, but not IgA, was detected in serum from which IgG was not depleted. Anti-human IgA-HRP detected IgA in both IgG-depleted and non-depleted samples.
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