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Charge and size of mesangial IgA in IgA nephropathy

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Charge and size of mesangial IgA in IgA nephropathy. To characterize the physicochemical properties of the mesangial IgA in primary IgA nephropathy, acid-eluates from percutaneous renal biopsies of 20 patients were examined. The acid-eluates were obtained from 1287 ± 498 glomerular sections. The IgA content (mean 15 ± 10 ng) represented 0.4% of the total eluted proteins. To analyze the molecular weight and the charge of eluted IgA, 11 eluates were subjected to high pressure liquid chromatography (at pH 6.8 and/or pH 3.5) and five eluates to isoelectric focusing on agarose. IgA was detected in the fractions by an IgA-RIA. Comparison of the elution profiles at different pH showed a statistically significant decrease of the excluded IgA peak (≥ 1,000,000 daltons), and a significant increase of polymeric IgA peaks (1,000,000–320,000 and 320,000 daltons) in acidic chromatography, as compared to non-dissociating conditions. Under acidic conditions, polymeric IgA represent 64% of total eluted IgA. Secretory component binding to polymeric IgA was demonstrated in four out of eight eluates tested. The isoelectric point (pI) of eluted IgA ranged from 4.5 to 5.6, contrasting with the broader and more neutral pI of normal serum IgA (4.5 to 6.8). This study shows that the multimeric nature of IgA, the formation of IgA complexes, and the anionic charge of IgA are likely to be involved in the mesangial IgA deposition in idiopathic IgA nephropathy.

Charge et poids moleculaire des IgA mésangiales au cours de la néphropathie à IgA. Les caractéristiques physico-chimiques des IgA mésangiales éluées à partir de biopsies rénales percutanées chez 20 malades atteints de la néphropathie à IgA ont été étudiées. Les eluats acides ont été obtenus à partir de 1287 ± 498 sections de glomérules. La moyenne de la quantité d'IgA éluées (15 ± 10 ng) représente 0,4% du total de protéines éluées. De façon à préciser le poids moléculaire et la charge des IgA éluées, 11 éluats ont été analysés après chromatographie liquide à haute pression (à pH 6.8 et/ou à pH 3.5) et cinq eluats ont été analysés par isoélectrofocalisation en agarose. Les IgA ont été détectés dans les fractions chromatographiques ou électrophorétiques par RIA-IgA. La comparaison des profils d'élution obtenus à pH acide et à pH neutre a montré une diminution significative des IgA présentes dans le pic d'exclusion (≥ 1,000,000 daltons) et une augmentation significative du pic des IgA polymériques (1,000,000–320,000 et 320,000 daltons). Ainsi, en conditions d'élution acide, 64% du total des IgA éluées sont sous forme polymérique. La fixation d'IgA polymériques sur le composant secrétoire a été démontrée dans quatre des huit éluats testés. Le point isoélectrique (pI) des IgA éluées varie de 4.5 à 5.6 correspondant à une restriction du pI des IgA mésangiales comparées aux IgA du sérum dont le pI varie de 4.5 à 6.8. Cette étude montre que la nature multimérique de l'IgA, la formation de complexes, ou d'aggrégats d'IgA et la charge anionique de l'IgA éluée du mésangium sont probablement impliquées dans la déposition mésangiale au cours de la néphropathie à IgA.

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IgA nephropathy is a form of glomerulonephritis (GN) characterized by mesangial granular deposits of IgA in patients presenting macroscopic or microscopic hematuria [1–3]. This disease is most common in young adults, predominantly in males [2]. It is the most frequent type of primary GN in several countries, with a prevalence ranging from 20 to 25% in southern Europe and Australia [4–6], up to 40% in Japan [7, 8]. In the long term, IgA nephropathy seems to progress to end-stage failure in about 25% of the patients, twenty years after the apparent onset [9].

The origin of mesangial IgA and the mechanism(s) responsible for its deposition remain unknown. The recurrence of deposited IgA in grafted kidneys [10], the elevation of serum IgA levels in about 30 to 50% of the cases [4, 5], a decrease of IgA specific suppressor T cell activity [11–13], and an increase of IgA specific helper T α cells [13, 14] suggest that the host immune response plays a role in the mechanism of the disease. Increase of polymeric forms of serum IgA, in these patients, has been reported [15–17], but could not be confirmed [18, 19]. No secretory component (SC) could be demonstrated along with the IgA deposited in the kidney by immunofluorescence studies [20]. However, the presence of polymeric IgA in the mesangium has been suggested by the in vitro fixation of the SC, although IgM could bind it also [21]. Dimeric IgA was demonstrated in eluates of open renal biopsy specimens from patients with IgA nephropathy [22].

Experimental models were first to suggest that only polymeric IgA or IgA immune complexes can be deposited in the mesangium [23, 24]. Recently, in experimental IgA nephropathy induced in mice after oral immunization, antigens, J chain, and IgA were detected in the mesangial area, showing that IgA is polymeric and probably derived from the secretory sites [25].

The purpose of this study was to analyze the physicochemical characteristics of mesangial IgA in primary IgA nephropathy. Since these properties can only be evaluated on soluble material, we first developed a micro-elution technique, which, coupled to a sensitive IgA-radioimmunoassay (RIA), allowed us to study the eluted material from routine percutaneous kidney biopsies.

Methods

Patients

Routine percutaneous renal biopsy specimens were obtained from 20 patients (13 males and seven females; mean age 31 ± 15 yrs, range, 14 to 69 yrs) with primary IgA nephropathy. The

diagnosis was made based upon clinical, immunofluorescence, and light microscopic findings. The patients had recurrent upper respiratory tract infections (9/20), macroscopic hematuria (8/20), hypertension (4/20), nephrotic syndrome (1/20), proteinuria < 3 g/24 hr (17/20). Ten patients were asymptomatic, and glomerulonephritis was discovered by routine analysis for proteinuria or hematuria. Seventeen of the 20 patients had normal renal function, and three of the 20 had moderate renal failure. None of them had clinical or biochemical evidence of systemic lupus erythematosus, liver disease, Henoch-Schönlein syndrome, or other systemic diseases.

Analyses

Light microscopic and immunofluorescence studies

Renal tissue was prepared for light microscopic studies as described previously [10]. For direct immunofluorescence studies, the specimens were frozen in isopentane cooled by liquid nitrogen and 3 μm sections were cut using a Slee cryostat. In each case, the following fluorescein isothiocyanate conjugated antisera were used on unfixed cryostat sections: anti-human γ, α, μ, C3, C4, Clq, fibrinogen, and albumin (Hyland Laboratories, Costa Mesa, California, USA, or Behringwerke A.G., Marburg an der Lahn, West Germany). The monospecificity of each antiserum was tested by immunodiffusion. Patients without tubular casts containing IgA, without IgA plasma cells in the interstitium, with few sclerotic glomeruli, and with strongly fluorescent IgA mesangial deposits were chosen deliberately. Four renal biopsy specimens from patients presenting hematuria and/or proteinuria, but in whom the kidneys were found to be normal by light and immunofluorescence microscopic analyses, were used as controls.

Micro-elution procedures

The elutions were performed on 20 renal biopsy specimens from patients with IgA nephropathy and on four normal renal biopsy specimens. Serial sections 3 μm thick of the specimens were prepared with a cryostat. The number of glomerular sections was determined by light microscopy every ten sections. Glomerular sections that ranged from 505 to 2300, with a mean ± SD = 1,287 ± 498, were used. The serial sections were incubated at 1 ml of phosphate buffered saline (PBS) in 1.5 cc microfuge tubes (Beckman Instruments, Fullerton, California, USA) for 5 min with gentle shaking at room temperature and centrifuged for 30 min (4,000*g*) at 4°C. This washing procedure was repeated 3 times and the pellet was resuspended and incubated for 4 hrs at 37°C in 200 μl of 0.02 M citrate buffer, pH 3.2 [26]. When indicated, 1 μg/ml aprotinin (Sigma Chemical Co, St. Louis, Missouri, USA) and 1 mM phenylmethylsulfonylfluoride (Sigma) were added at 0 and 2 hrs, to prevent proteolysis during the elution. After elution, the renal tissues were centrifuged (4,000*g* for 30 min) and the eluates were neutralized with 40μl of 1 M Na_2HPO_4 and stored at −80°C. The total protein concentration in the eluates was measured using the Bradford method [27] (BIO-RAD Chemical Division, Richmond, California, USA).

Purification of IgA

Briefly, highly purified IgA was obtained from normal human sera (NHS) by euglobulin precipitation (2.5 mM EDTA, 5 mM phosphate buffer, pH 5.9) and DEAE ion-exchange chromatography (Whatman, London, England), followed by passage through a Sephadex G 200 column equilibrated with PBS. The pooled IgA fractions passed over Sepharose 4B-anti-human IgG. The purified IgA contained over 95% IgA as assessed by immunodiffusion, immunoelectrophoresis using anti-whole human serum antiserum (Hyland) and by SDS-polyacrylamide gel electrophoresis using 5.6% acrylamide gels. The IgG content was less than 0.1% measured by immunonephelometry (Hyland).

Chromatographic analysis

Eleven eluates were subjected to high pressure liquid chromatography (HPLC) on columns, 7.5 mm ID × 30 cm, of Spherogel TSK G 4,000 SW dp 10 μ (Altex Scientific Inc., Berkeley, California, USA; with 5,000 to 1,000,000 dalton fractionation range). 250 μl were applied to the HPLC column with a flow rate of 0.1 ml/min, and 250 μl fractions were collected in 1% ovalbumin-PBS coated tubes. The column was standardized with molecular weight markers (acid treated under the same conditions as the eluates): free $Na^{125}I$, IgA, monoclonal dimeric IgA, and dextran blue (2×10^6 daltons). Six eluates were analyzed in 0.1M phosphate buffer, pH 6.8 containing 0.1 M NaCl; three eluates were mixed with 20μl of 1M citric acid, chromatographed in 0.02 M citrate buffer, pH 3.5 containing 0.1 M NaCl and neutralized after HPLC with 30μl of 1M Na_2HPO_4; and two eluates (Nos. 7 and 8) were divided into two samples and analyzed using both buffers. Monoclonal dimeric IgA was provided by Dr. S. Iscaki (Inst. Pasteur, Paris, France) [28].

Isoelectric focusing (IEF)

Five eluates were analyzed on agarose gels (1.5 mm) containing 0.8% agarose (agarose EF, LKB Instruments, Inc., Gaithersburg, Maryland, USA), 10% sorbitol and 2.5% ampholyte (ampholine 3.5 to 9.5, LKB). The mixture was dissolved in double distilled water and poured onto a gelbond covered glass plate (LKB) and kept overnight at 4°C. All focusing was performed on an Ultraphor apparatus (LKB) cooled by a thermostatically regulated water bath set at 10°C. The anolyte was 0.5 M acetic acid and the catholyte 0.5 M sodium hydroxide. A 140μl aliquot from each sample, previously dissociated with 10 μl of 1 M citric acid, was applied in the middle of the agarose gel. Purified IgA and NHS (acid treated under the same conditions as the eluates) were analyzed by IEF as controls. The proteins were then focused with a constant power of 10 W for 1.5 hrs. The gel was cut into 0.5 cm × 1 cm gel slices [29] and incubated overnight at 4°C in 0.2 ml NaCl-EDTA-Tris buffer (150mM NaCl, 5 mM EDTA, 50 mM Tris and 0.02% sodium azide, pH 7.0) containing 1% ovalbumin and 0.05% Nonidet P-40 (NP-40, Shell Chemical Co., London, England) (NP40-OVA-NET buffer). Direct pH measurements were made with control slices incubated in water. Anodic and cathodic fractions were neutralized to pH 7.0 with 1 M Tris or 1 M HCl, respectively.

IgA radioimmunoassay

IgA was detected in the HPLC and IEF fractions by RIA using rabbit anti-human α chain antibody (Dakopatts, Copenhagen, Denmark) and normal polyclonal IgA, purified as described above, was radioiodinated with $Na^{125}I$ (Amersham,

Buckinghamshire, England) for each assay, by the chloramine T method [30]. The specific activity was 8×10^7 cpm/μg. Formalin-fixed staphylococcus aureus (staph A) (Bethesda Research Laboratories, Inc., Gaithersburg, Maryland, USA), pretreated in 10% β-mercaptoethanol and 3% SDS at 95°C for 30 min, and washed in PBS and in NP40-OVA-NET buffer, was used to precipitate anti-IgA antibodies [31]. The inhibition of ^{125}I-IgA binding to anti-IgA antibodies by the unknown sample was determined after precipitation with staph A and the sensitivity of this IgA-RIA was 0.02 ng, (50% inhibition ranged between 0.07 and 0.12 ng). The antibody was considered monospecific for the determination of IgA levels because IgG and IgM failed to inhibit the ^{125}I-IgA binding at concentrations 2×10^4 times greater than the sensitivity.

Secretory component binding assay

The binding of purified human colostrum SC was assessed in the HPLC fractions corresponding to the IgA peaks. The SC was ^{125}I-labeled by the Bolton and Hunter reagent (Amersham) with a specific activity of 1×10^6 cpm/μg. HPLC fractions were incubated sequentially with ^{125}I-SC and anti-α chain antibody for 1 hr at 37°C. The binding of SC to IgA was detected by the precipitation of the ^{125}I-SC-IgA-anti-α chain complexes by staph A in NP40-OVA-NET buffer. The results were calculated according to the following formula:

$$\text{Index of SC binding} = \frac{\text{experimental bound cpm} - \text{background cpm}}{\text{buffer bound cpm} - \text{background cpm}}$$

The assay was considered positive when the index was ≥ 2 (0.1 ng of human monoclonal dimeric IgA corresponds to a SC binding index of 2.1). Purified SC was provided by Dr. S. Iscaki (Inst. Pasteur, Paris, France) [28].

Results

Pathological findings

In immunofluorescence studies, IgA deposits were found predominantly in the mesangial area and were brightly stained. Eleven out of the 20 patients had small amounts of IgM, while 16 of them had small amounts of IgG in association with the IgA deposits. C3 was present in the mesangium like IgA, but was less intensely stained.

Light microscopic studies revealed mesangial enlargement in all patients. Seven out of the 20 cases had slight focal mesangial hypercellularity. Four of the patients presented diffuse moderate mesangial proliferation. Nine cases exhibited segmental lesions characterized by either sclerosis or epithelial proliferation.

Protein and IgA contents of the eluates

The total amounts of eluted proteins were comparable in four patients and in four normal kidneys (4.3 ± 0.93 μg from 1386 ± 431 glomerular sections and 4.7 ± 1.2 μg from 1590 ± 401 glomerular sections, respectively). IgA amounts (mean ± SD) in eleven eluates prepared from IgA nephropathy biopsy specimens were 15 ± 10 ng (ranging from 0.7 to 28 ng), equivalent to about 0.4% of the total eluted proteins. In contrast, no IgA was detected in four normal renal eluates, thus demonstrating that the washes prior to elution removed serum proteins effectively. The amounts of eluted IgA did not correlate with the size and intensity of the deposits analyzed by immunofluorescence in a double blind study, according to linear regression analysis (r = 0.45, NS).

Table 1. Molecular weight distribution of mesangial IgA after high pressure liquid chromatography at pH 6.8 and/or pH 3.5

MW, × 10^3 daltons	% of eluted IgA under		
	Neutral	Acidic	P[a]
<160	27 ± 18	22 ± 12	NS
160	18 ± 12	10 ± 7	NS
320	18 ± 15	37 ± 12	< 0.05
320 to 1000	5 ± 6	27 ± 23	< 0.05
≥1000	29 ± 16	5 ± 7	< 0.01

[a] Significant differences (Student's t test) were found between different molecular weights under both conditions.

Molecular weight analysis and SC binding of eluted IgA

Eleven eluates were used to separate IgA according to molecular weight by high pressure liquid chromatography. Fractions from eight of these eluates were then divided into two samples: one for IgA detection by IgA-RIA and the other for the SC binding assay. Since the three remaining eluates contained insufficient amounts of IgA for both assays, only the IgA-RIA was performed. The percentages of IgA molecular forms were determined by profile integration after HPLC. Table 1 shows the results obtained with eight eluates analyzed under non-dissociating conditions (pH 6.8) and five eluates subjected to acidic chromatography, at pH 3.5. Comparison of the molecular weights of eluted IgA after pH 6.8 and pH 3.5 HPLC column shows a decrease of the excluded IgA peak (≥ 1,000,000 daltons) and an increase of 1,000,000–320,000 and 320,000 dalton IgA peaks (64% of total eluted IgA) after acidic chromatography, with statistically significant differences according to the Student's t test ($P < 0.01$ and $P < 0.05$, respectively). The IgA monomer peak (160,000 daltons) represented 10 to 20% of the total IgA content in both analyses, but showed no significant differences. For two eluates (Nos. 7 and 8), the amounts of eluted IgA were sufficient to be analyzed under both buffer conditions (Fig. 1). Elution profiles at acid pH differ from non-dissociating conditions, mainly by the decrease of the large molecular weight (≥ 1,000,000 daltons) IgA peaks and the predominance of the dimeric IgA forms. The low molecular weight compounds, smaller than 160,000 daltons, were found in both cases, as well as the presence of protease inhibitors in six patients analyzed. There were no significant statistical differences between elutions without (elutions 1 to 5) or with (elutions 6 to 11) protease inhibitors (mean, 27 ± 17 or 22 ± 12%, respectively). The affinity of eight eluates for purified SC was analyzed after HPLC. In four eluates, SC binding IgA were found in polymeric forms. Patient eight's eluate was analyzed under both conditions, and the dimeric IgA fractions (320,000 daltons) bound the SC only after dissociating chromatography (Fig. 1). Neither monomers nor large molecular weight IgA bound the SC. Fifty percent of the eluates did not bind any SC under the same conditions.

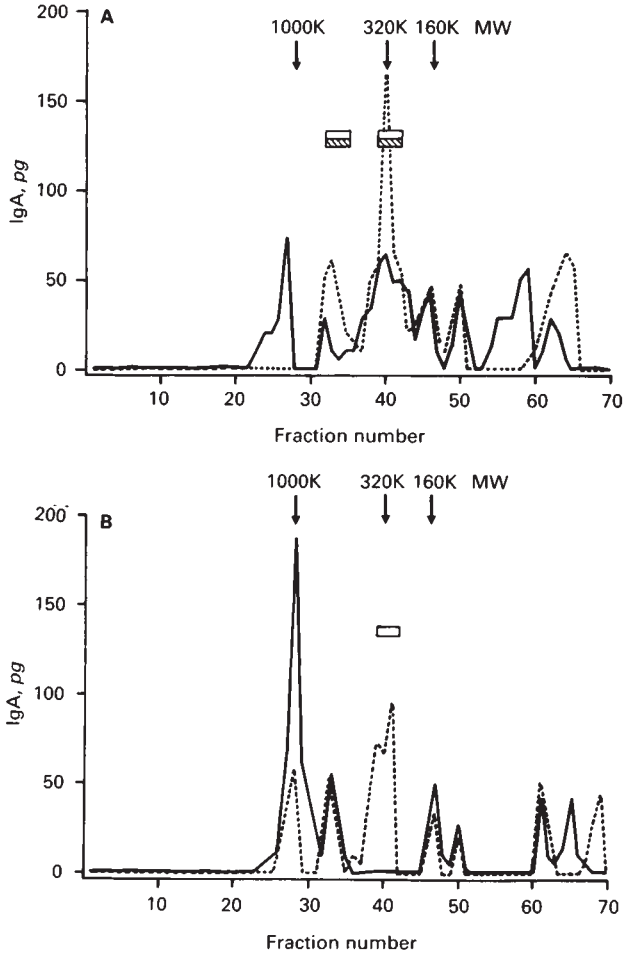

Fig. 1. A *HPLC elution profiles of mesangial IgA (Patient 7), using two different pH buffers (pH 6.8 (—) and pH 3.5 (-----)).* IgA was detected in the fractions by an IgA-RIA. SC binding was determined in the IgA peaks, described in **Methods.** Positive binding is shown at both elution conditions (pH 6.8 (▨) and pH 3.5 (□)). **B** *HPLC elution profiles of mesangial IgA (Patient 8), as described above.*

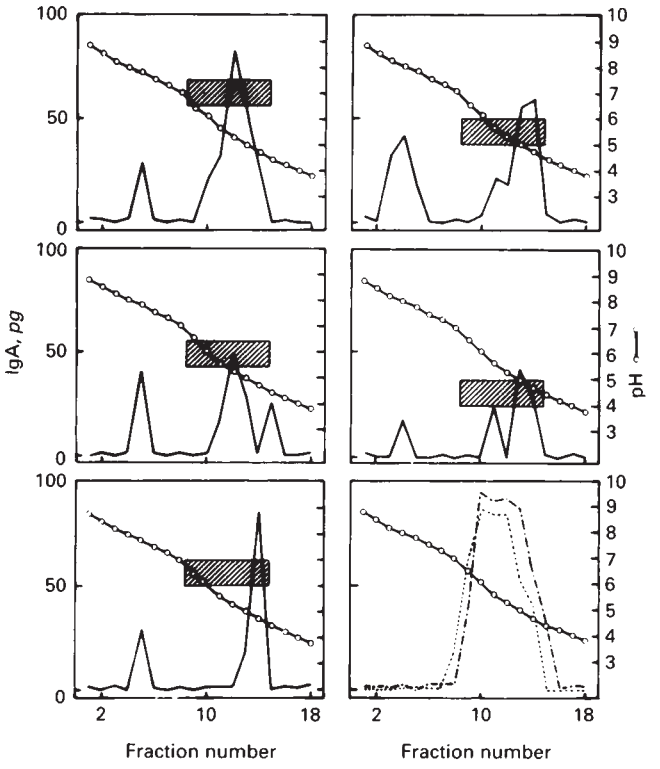

Fig. 2. *Charge distribution of mesangial IgA analyzed by isoelectric focusing on agarose gels.* Focusings were performed in the pH range 3.5 to 9.5, described in **Methods.** The gel was sliced and proteins were eluted. The IgA was detected in the eluates by an IgA-RIA; five patients (—); highly purified serum IgA (----); and IgA detected in NHS (--). The hatched area (▨) represents the normal isoelectric range of serum IgA.

Charge characterization of mesangial IgA

The charge of eluted IgA was analyzed for five patients by isoelectric focusing on agarose. After migration, the gel slice eluates were tested for their IgA contents by IgA-RIA. Figure 2 shows the isoelectric points (pI) of eluted IgA for all patients and normal serum IgA (purified serum IgA and NHS). The pI of eluted IgA ranged from 4.5 to 5.6, contrasting with the more heterogeneous pI of purified serum IgA and IgA detected in NHS treated under the same conditions as the eluates (4.7 to 6.8 and 4.5 to 6.6, respectively). A small basic IgA peak was observed for all eluates studied, with pI between 7.8 and 8.0, varying from 13 to 35% of the IgA peaks, determined by profile integration.

Discussion

We have developed a micro-elution technique, which allows the recovery of nanogram quantities of mesangial IgA, determined by a sensitive IgA-RIA, using routine percutaneous renal biopsy specimens obtained from IgA nephropathy patients. Cases presenting only mesangial IgA deposits were chosen in order to elucidate the factors involved in the mesangial IgA deposition. The amount of eluted IgA was equivalent to 0.4% of the total proteins present in the eluates, which were primarily kidney tissue proteins removed by the acidic treatment.

The predominant molecular weight of the mesangial IgA was polymeric (≥ 320,000 daltons). Under neutral chromatography, 29% of the eluted IgA was high molecular weight IgA complexes (≥ 1,000,000 daltons), as described previously [32]. In contrast, under acidic conditions these IgA complexes were mainly dissociated into dimeric fractions (320,000 daltons), corresponding to 37% and constituting the major molecular form of the eluted IgA. This is consistent with the results of Tomino et al [22], who found mainly dimeric IgA in eluates of open surgical biopsy specimens.

In an attempt to prevent possible proteolysis by low pH-active tissue peptidases, we examined six elutions in the presence of protease inhibitors. There were no significant differences between the elutions performed with or without these inhibitors. Indeed, IgA cleavage did not occur during the elution process, since the addition of intact ^{125}I-IgA at the time of elution, in the presence or absence of protease inhibitors, did not lead to the degradation of the labeled IgA. Thus, the small IgA peptides found in this study (50,000 to 5,000 daltons) probably result from IgA degradation either in vivo by mesangial [33, 34] or circulating cell enzymes, or during the time between the biopsy and freezing.

Secretory component binding capacity was assessed in the HPLC fractions containing IgA. Four of the eight eluates tested contained polymeric IgA with SC binding activity. It is possible that the four negative eluates contained mainly polymeric IgA which lacked the J chain [35]. This form is known to have no affinity for SC at physiological ionic strength. On the other hand, the SC binding assay may not be sensitive enough to detect minute quantities of polymeric IgA (< 0.1 ng) in the HPLC fractions. After chromatography, it was shown that only polymeric but not monomeric IgA bound SC. The larger IgA peak eluted in the void volume did not consistently bind SC, possibly because of inhibition by IgM often present in the glomeruli, or as a result of a particular arrangement of the high molecular weight IgA complex restricting SC access.

These results suggest that mesangial IgA is composed mainly of polymeric and dimeric IgA, representing 64% of the total eluted IgA, with affinity for SC. Furthermore, this percentage is probably underestimated, since the RIA was standardized using monomeric IgA and did not take the IgA size into account [36]. However, it is not known from this study whether IgA antibody activity (anti-antigens or anti-idiotypes) is involved in the formation of complexes. This could be suggested indirectly by the dissociation of the eluted high molecular weight mesangial complexes under acidic conditions, although they may also be IgA aggregates.

The isoelectrofocusing study of the mesangial IgA revealed a similar pattern for the five biopsy specimens studied. The eluted IgA was predominantly anionic, with a pI ranging from 4.5 to 5.6. On the other hand, highly purified IgA or IgA present in normal serum, treated under the same acidic conditions as the eluates, showed a broad range of pI (4.5 to 6.8). In each eluate, a small amount of highly "basic IgA" (pI 7.8 to 8.0) was also found. Whether this represents abnormal IgA, IgA fragments, or IgA covalently bound to a basic compound cannot be determined from this study.

The restricted pI of eluted IgA suggests that electrostatic binding may play a role in mesangial IgA deposition. In the majority of cases, no mesangial IgA deposits remained after elution, as assessed by immunofluorescence, thus excluding a selection of an IgA subpopulation of low pI by the acidic treatment.

The predominant anionic nature of the mesangial IgA in IgA nephropathy may be due to a particular carbohydrate composition or to a high content of acidic amino acids in the variable region.

Our findings are consistent with numerous experimental models that show molecular size and charge-dependent glomerular deposition. The mesangial matrix has been shown to entrap large circulating soluble macromolecules, such as aggregated human IgG or large preformed immune complexes [37–40]. The negative charge of the eluted IgA from patients with idiopathic IgA nephropathy that we observed is reminiscent of experimental models in which anionic antibodies, antigens, or immune complexes were deposited in the mesangium [41–43].

In conclusion, although the origin of the deposited IgA and its antibody activity in patients with primary IgA nephropathy are still unclear, this study suggests three conditions that may favor mesangial IgA deposition: *1)* the multimeric nature of IgA; *2)* circulating or in situ IgA complex formation; and *3)* the anionic charge of IgA.

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