NEPHRON

A.S. Shuib [a]
C.T. Chua [b]
O.H. Hashim [a]

Departments of
[a] Biochemistry and
[b] Medicine, Faculty of Medicine,
University of Malaya,
Kuala Lumpur, Malaysia

Sera of IgA Nephropathy Patients Contain a Heterogeneous Population of Relatively Cationic Alpha-Heavy Chains

Abstract
Sera of IgA nephropathy (IgAN) patients and normal subjects were analysed by two-dimensional (2-D) gel electrophoresis. Densitometric analysis of the 2-D gels of IgAN patients and normal subjects revealed that their protein maps were comparable. There was no shift of pI values in the major α-heavy chain spots. However, the volume of the α-heavy chain bands were differently distributed. Distribution was significantly lower at the anionic region in IgAN patients (mean anionic:cationic ratio of 1.184 ± 0.311) as compared to normal healthy controls (mean anionic:cationic ratio of 2.139 ± 0.538). Our data are in support of the previously reported findings that IgA1 of IgAN patients were lacking in sialic acid residues.

Introduction

IgA nephropathy (IgAN), a disease of unknown etiology, is characterised by the predominant deposition of IgA1 in the renal mesangia of all the patients. Elevation of the serum levels of IgA1, IgA1 immune complexes and IgA1 rheumatoid factor was also detected in approximately half of the patients [1]. Several recent studies have associated the etiopathogenicity of IgAN with abnormal hinge-glycosylation of IgA1. The hinge region O-glycans of IgA1 are involved in the asialoglycoprotein receptor-mediated hepatic uptake mechanism [2]. In patients with IgAN, it was postulated that defects in post-translational O-glycosylation, or structural modification of the O-glycans of IgA1, may subject the immunoglobulins to a change in their pattern of clearance and their consequential deposition in the glomerular mesangium [3].

Among the reported abnormalities of O-linked carbohydrate structures of IgA1 in IgAN patients were a general decrease in terminal galactosylation of the hinge region O-linked carbohydrate moieties [4, 5] and a significant shift of monosialylation of Galβ1-3GalNac of the O-glycans to asialo-Galβ1-3GalNac moieties [6, 7]. In addition, the λ-containing IgA1 of IgAN patients were shown to be more negatively charged [8–11], and higher association of λ than κ light chains with IgA1 of IgAN patients has been observed [12–14]. The unusually glycosylated IgA from IgAN patients and IgA1 with modified O-glycan structures were also demonstrated to have increased binding to mesangial cells [15, 16].

In the present study we analysed the α-heavy chains of IgAN patients and normal healthy controls by subjecting their sera to high-resolution two-dimensional (2-D) electrophoresis and computerised densitometry.

Dr. O.H. Hashim
Department of Biochemistry
Faculty of Medicine, University of Malaya
50603 Kuala Lumpur (Malaysia)
E-Mail onnhashim@medicine.med.um.edu.my

Materials and Methods

Sera

Serum samples were obtained from IgAN outpatients (age range 22–58 years) of the University Hospital, Kuala Lumpur, and normal healthy adult volunteers (age range 20–42 years). The subjects came from different ethnic backgrounds (Malay, Chinese and Indian).

Six millilitres of blood were collected in LIP-VAC gel serum separator (L.I.P., West Yorkshire, UK) tubes. The tubes were centrifuged at 3,000 *g* for 10 min, and separated sera were collected and kept at –20°C.

Lectin

Preparation of crude extract of champedak seed lectin was performed as previously described [15]. The crude extract of lectin was subjected to an immobilised galactose-Sepharose affinity chromatography in 0.01 *M* phosphate buffer containing 0.15 *M* NaCl, pH 7.2 (PBS). Elution of champedak lectin-C was carried out by using 0.8 *M* of *D*-galactose dissolved in PBS. The lectin was then exhaustively dialysed against PBS.

Conjugation of champedak lectin-C to horseradish peroxidase (HRP) was performed by incubation of the purified lectin with periodate-activated HRP in sodium carbonate buffer (pH 9.5) for 2 h at room temperature. The solution was stirred for another 2 h at 4°C upon addition of sodium borohydrate to reduce the remaining free enzymes. The conjugated lectin was kept at 4°C in the presence of 60% (v/v) glycerol.

2-D Gel Electrophoresis

2-D gel electrophoresis was performed using the Multiphor II Electrophoresis System based on the recommended method by Pharmacia (Uppsala, Sweden). Pre-cast immobiline dry strips pH 4–7 (Pharmacia) were rehydrated overnight in solution consisting of 8 *M* urea, 0.5% (v/v) Triton X-100, 0.5% (v/v) Pharmalyte 3–10 and 12 m*M* dithiothreithol (DTT). The strips were aligned in a tray that was filled with silicon oil. Serum was diluted four times with sample buffer consisting of 9 *M* urea, 60 m*M* DTT, 2% (v/v) Pharmalyte 3–10 and 0.5% (v/v) Triton X-100. Isoelectric focusing was performed in 3 steps by varying the voltage as suggested by the manufacturer.

For the second dimension, the strips were incubated for 10 min in equilibration solution consisting of 6 *M* urea, 1% (w/v) SDS and 30% (v/v) glycerol dissolved in 0.05 *M* Tris-HCl (pH 6.8), in the presence of 0.3 *M* DTT. This was followed by incubation in similar equilibration solution containing 50 m*M* iodoacetamide for another 10 min. Strips were placed on a SDS-PAGE gradient gel 8–18 (Pharmacia) and electrophoresed for 95 min. The gel was developed by staining with Coomassie brilliant blue R-250 and dried. Calibration of the isoelectric points (pI) of the gels was performed by using the 2-D SDS-PAGE standards (Bio-Rad, Hercules, Calif., USA).

Western Blotting

NovaBlot Kit of Multiphor II Electrophoresis System (Pharmacia) was used for transferring the protein to nitrocellulose membrane (0.2 µm). The unit was run at 0.8 mA/cm² for 1 h. The nitrocellulose was incubated in Tris-buffered saline (TBS, pH 7.5) in the presence of 3% (w/v) bovine serum albumin for 1 h at room temperature. The membrane was washed three times with TBS with 0.1% (v/v) tween-20 (pH 7.5) and subsequently incubated in HRP-conjugate diluted/dissolved in the same buffer (2,000 times dilution of anti-human IgA,

Binding Site, Birmingham, UK; or 2 mg/ml of champedak lectin-C) for another 1 h. Development of the Western blot was performed using 25 ng 3,3′-diaminobenzidine and 5 µl 30% (v/v) H_2O_2 in 50 ml TBS. The reaction was stopped by washing the membrane with distilled water.

Data Analysis

Dried 2-D electrophoretic gels and Western blots were scanned by using Model GS-690 Imaging Densitometer and analysed by using the computerised Molecular Analyst densitometry software (Bio-Rad, Hercules, Calif., USA). Distribution of specific regions of the α-heavy chain band was analysed in terms of volume, i.e., optical density ×1 area in square millimetres of region. The Mann-Whitney U test was used for estimating statistical significance. A p value of <0.05 was considered to be a significant difference.

## Results

2-D Electrophoretic Analysis of Serum Samples

Complex protein maps showing hundreds of spots were obtained when serum samples were analysed by high-resolution 2-D gel electrophoresis with Coomassie blue staining (fig. 1). Altogether, the 2-D separation analysis was performed on sera of 12 normal subjects and 12 IgAN patients, and their Coomassie-stained gels were scanned by using an imaging densitometer. Analysis of the gels conducted by using the computerised Molecular Analyst software failed to detect any significant difference between the protein maps obtained from normal subjects and IgAN patients.

To locate the position of the α-heavy chain, the 2-D gels were blotted onto a nitrocellulose membrane and detected with enzyme-linked α-heavy chain-specific anti-human IgA (fig. 2). The α-heavy chain band detected from the Western blot analysis was divided into the anionic (pI <5.6) and cationic (pI >5.6) regions according to previously adopted methods [8, 9, 11]. When compared with the Coomassie-stained 2-D gels that were subjected to densitometric scanning and analysis, it was obvious that the Coomassie-stained 2-D separation was only capable of resolving the anionic region of the α-heavy chain band as the cationic region of the band was superimposed with several other protein spots.

Within the anionic region of the α-heavy chain band, five spots which vary in volume and could be clearly distinguished according to their charges were detected. These were denoted as S1 (pI 5.05), S2 (pI 5.13), S3 (pI 5.25), S4 (pI 5.35), and S5 (pI 5.49), respectively, from acidic to basic sides of the gel. For comparative purposes the anionic region of α-heavy chain band was further divided into the acidic (pI <5.25) and basic (pI >5.25)
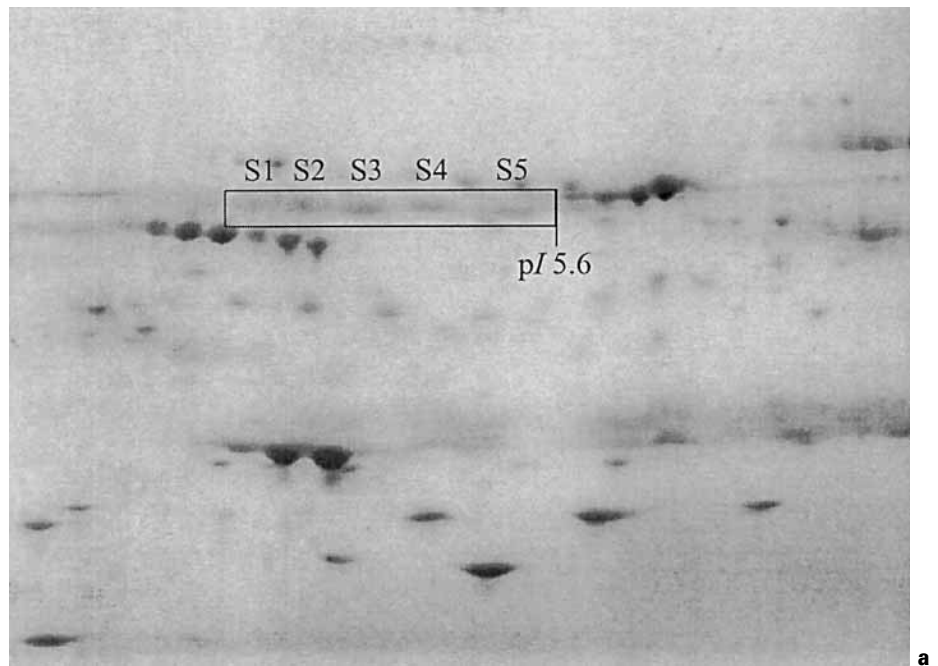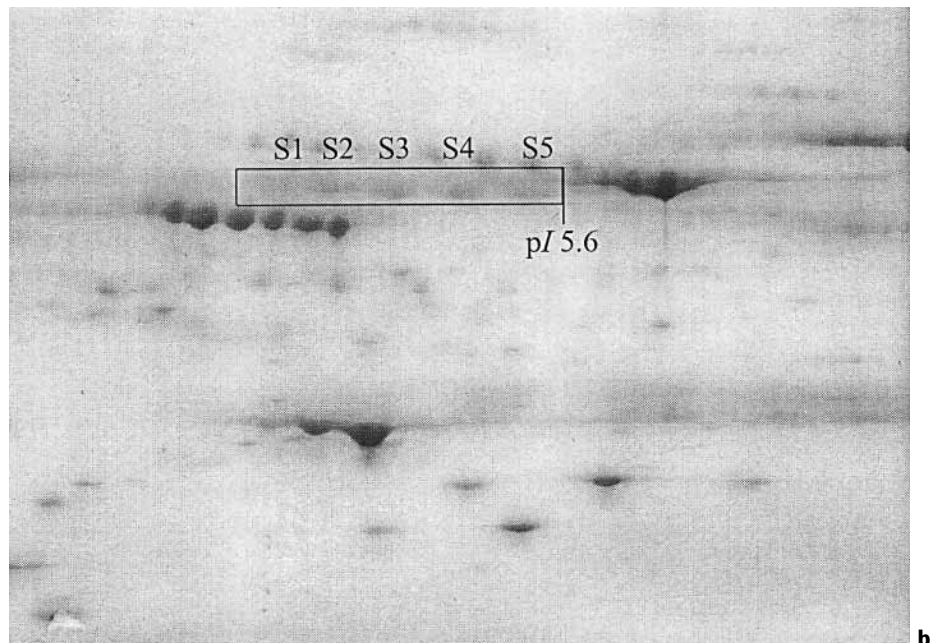
Fig. 1. High resolution 2-D electrophoretic map of human serum. **a** Normal subject. **b** IgAN patient. Gels were stained with Coomassie blue. The major anionic α-heavy chain spots were enframed and denoted as S1 (pI 5.05), S2 (pI 5.13), S3 (pI 5.25), S4 (pI 5.35), and S5 (pI 5.49) from the acidic to basic sides of the gel. The acid side on all gels and blots is to the left and the relative molecular mass declines from their top.

Fig. 2. Western blot analysis using antiserum. To determine the location of the α-heavy chains, 2-D gel of normal human serum was blotted onto nitocellulose and detected with the HRP-conjugated polyclonal α-chain-specific, anti-human IgA. A dividing pI of 5.6 was used to separate the anionic and cationic regions of the band.
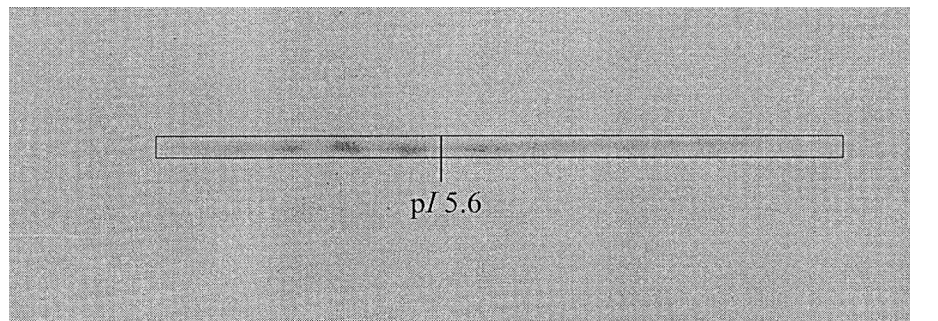
sides. A dividing pI of 5.25 was chosen for its being in the middle of the range. The ratio of volume contribution of the acidic to basic sides of the anionic region of the α-heavy chain band was subsequently calculated. Analysis performed on serum samples of normal healthy subjects (n = 12) and IgAN patients (n = 12) demonstrated that there was no significant difference between their mean ratio of volume distribution (fig. 3).

Champedak Lectin-C Western Blot Analysis

Since Coomassie-stained 2-D gels allowed limited densitometric analysis of the α-heavy chain band only to its anionic region, we subjected the 2-D gels of sera of the normal subjects and IgAN patients to Western blotting. In this study we used enzyme-conjugated champedak lectin-C, a lectin which has been shown to demonstrate selective strong interaction with O-glycans of IgA1 [17, 18], to detect the α-heavy chain band. Examples of the 2-D lectin-C-detected Western blots of sera of normal controls and IgAN patients are shown in figure 4. Our results demonstrate that champedak lectin-C was a better and more sensitive detecting reagent for Western blotting of the α-heavy chain spots as compared to the α-heavy chain-specific anti-human IgA polyclonal antiserum that was earlier used. The pI of the α-heavy chain from IgAN patients and healthy controls resolved by this method
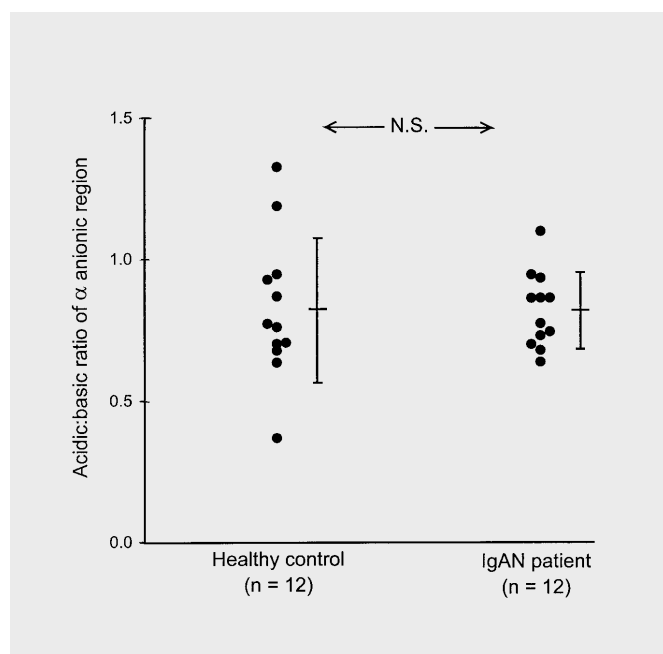

Fig. 3. Mean acidic:basic ratio of the anionic region of the α-heavy chain from normal controls and IgAN patients. A mid-range pI of 5.25 was used to divide the anionic region into its acidic and basic sides. There was no significant difference between the mean ratio of volume distribution of acidic:basic sides of the anionic region of the α-heavy chains from normal controls (0.825 ± 0.255) and IgAN patients (0.820 ± 0.133).

Fig. 4. Champedak lectin-C Western blot analysis. 2-D gels were blotted onto nitocellulose and detected with HRP-conjugated champedak lectin-C. Since lectin-C interacted with O-linked Galβ1-3GalNAc carbohydrate structures, other O-glycan-containing glycopeptides besides the α-heavy chains of IgA1 were also detected. Densitometric analysis was, however, focused only on the α-chain spots of IgA1 (enframed). Western blot analysis was performed on the sera of a normal subject (**a**) and an IgAN patient (**b**). The anionic and cationic regions of the blotted α-heavy chains were separated at the mid-range pI or 5.6.
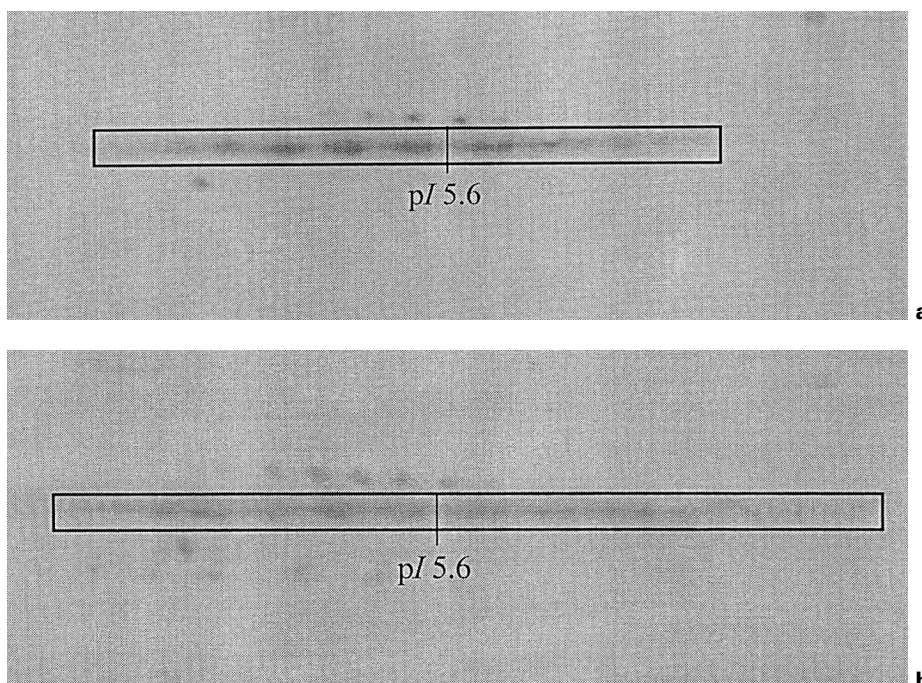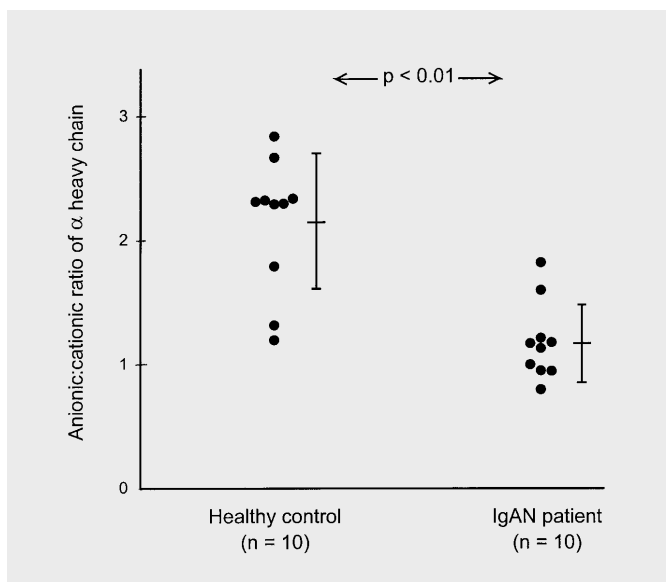
Fig. 5. Mean anionic:cationic ratio of the total α-heavy chain band from normal healthy controls and IgAN patients. A pI of 5.6 was used to divide the α-heavy chain band into its anionic and cationic regions. The mean ratio of patients (1.184 ± 0.311) was significantly lower than normal healthy controls (2.139 ± 0.538).

spanned from 4.4 to 6.8, a result which is compatible with several previously reported studies on IgA [10–12].

By using the IgA1-binding lectin as probe, the α-heavy chain band was fully resolved without any interference of the other serum proteins at its cationic region. It was thus possible to analyse the ratio of the volume distribution of the anionic:cationic region of the band using a pI of 5.6 as the dividing point according to the previously reported method [8, 9, 11]. Figure 5 demonstrates the data of analysis performed on the serum samples of normal subjects (n = 10) and IgAN patients (n = 10). The mean anionic:cationic α-heavy chain volume distribution ratio was significantly higher in normal healthy controls (2.139 ± 0.538) than the mean ratio of IgAN patients (1.184 ± 0.311).

Discussion

The main objective of this study was to compare IgAN patients and normal healthy subjects by separation of their serum proteins using high-resolution 2-D gel electrophoresis. The method provided better resolution of glycoproteins varying in charge distribution as compared to the conventional isoelectric focusing in a single dimension.

Instead of staining the 2-D gels with silver as normally done, Coomassie blue was used in this study. This was because silver stain gave higher background which affected the scanning process. Despite the sensitivity of silver in picking up a low concentration of proteins, the patterns of the α-heavy chain spots that were resolved in Coomassie-stained gels were similar and adequately intense for analytical purposes.

Comparably similar protein maps were obtained from the 2-D electrophoretic separation of sera of normal healthy subjects and IgAN patients. Because of the presence of other proteins at the cationic region of the α-heavy chain band, direct densitometric analysis of the Coomassie-stained gels was only made possible at the anionic region (pI <5.6). Within this region, the α-heavy chain was clearly resolved into five separate spots in all sera that were studied. There was no shift of pI values in the major anionic α-heavy chain spots of IgAN patients as compared to normal controls.

Since the five major spots at the anionic region of the α-chain band were clearly distinguishable in all sera that were tested, an attempt was made to determine whether there was a difference in the pattern of α-heavy chain distribution among the spots obtained from healthy controls and IgAN patients. The anionic region of the α-heavy chain band was thus further divided into the acidic and basic sides using the mid-range pI of 5.25 as the dividing factor. However, there was no significant difference between their mean ratio of volume distribution of acidic:basic sides of the anionic region of the α-heavy chain bands of normal healthy controls and IgAN patients.

A more complete analysis of the 2-D gel separation of sera of normal subjects and IgAN patients was made possible when the gels were Western-blotted onto a nitrocellulose membrane and detected with an enzyme-conjugated IgA1-reactive champedak lectin-C. Since the Western blots were no longer encumbered with the other serum proteins at the cationic region of the α-heavy chain band, it was possible to obtain the anionic:cationic volume distribution ratio of the band. Analysis performed on sera of IgAN patients (n = 10) demonstrated an anionic:cationic α-heavy chain mean distribution ratio of 1.184 ± 0.311, which was significantly lower than the mean anionic:cationic ratio of 2.139 ± 0.538 obtained from sera of normal healthy subjects (n = 10).

The above differential charge distribution generally imply that the α-heavy chains of IgAN patients were more cationic and thus lacking in sialic acid as compared to the α-heavy chain of normal healthy controls. This is compat-

ible with the recent report of Hiki et al. [7] which demonstrated that the IgA1 of IgAN patients contained a relatively higher concentration of asialo-Gal-GalNAc moieties and was lacking in monosialo-Gal-GalNAc structures when compared with the IgA1 from healthy controls. On the other hand, studies by isoelectric focusing have also demonstrated that the λ-light chain containing IgA of IgAN patients were more anionic [9–11]. The anionic nature of intact whole IgA molecules may be attributed to the presence of the λ-light chains since κ-containing IgA1 were shown to be cationic in nature. In this study, we only focused on the pI of the glycosylated α-heavy chains instead of the total intact IgA1 molecules.

The data of this study provide additional experimental evidence to demonstrate the existence of carbohydrate structural differences between IgA1 on IgAN patients and normal subjects. The question of how these changes would relate to the underlying pathogenesis of IgAN is unclear. The unusual structure of the O-glycans of IgA1 in IgAN patients may induce a conformational instability to the IgA1 molecule as proposed by Hiki et al. [7], and/or cause a glomerular accumulation of IgA1 as suggested by Mestecky et al. [16]. The latter proposal was based on the observation that modification of the structures of carbohydrate moieties of IgA1 with neuraminidase and β-galactosidase caused a decrease in binding to the liver cells but an increase in binding to the mesangial cells of the kidney.

Acknowledgements

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