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Circulating IgA, IgG, and IgM class antibody against *Haemophilus parainfluenzae* antigens in patients with IgA nephropathy

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SUMMARY

We previously demonstrated a close relationship between the outer membranes of *Haemophilus parainfluenzae* (HP) antigens (OMHP) and IgA nephropathy (IgAN). Our objective was to clarify the relationship among IgA, IgG, and IgM class antibody against OMHP in the sera of 44 patients with IgAN and 62 patients with other glomerular diseases (OGD) by ELISA. Patients with IgAN showed a significantly higher level of IgA antibodies ($P < 0{\cdot}0005$) and IgG antibodies ($P < 0{\cdot}001$) against OMHP, than did patients with OGD. Positive correlations were observed between IgA and IgG antibodies, between IgA and IgM antibodies, and between IgG and IgM antibodies against OMHP in the sera of patients with IgAN. Immunoblotting showed that IgA, IgG, or IgM antibodies against OHMP in the sera of all patients with IgAN bound to the components of OMHP. Amino acid sequences of three components of OMHP recognized by the sera from patients with IgAN revealed homology with those reported for outer membrane protein (OMP) P6 precursor, OMP P5, and P2 porin protein of *H. influenzae*. Results suggest that patients with IgAN have glomerular deposits of OMP P6 precursor, OMP P5, or P2 porin protein of HP, and a specific increase in the production of IgA antibodies against OMHP via polyclonal activation against these, with switching of production from one isotype to another, e.g. from IgM to IgA.

Keywords *Haemophilus parainfluenzae* IgA nephropathy outer membrane class switch

INTRODUCTION

IgA nephropathy (IgAN), a common glomerular disease, is characterized by the presence of IgA deposits, predominantly in the glomerular mesangium, and by mesangial proliferative glomerulonephritis (GN) [1]. Concerning its pathogenesis, several investigators suggest that the deposited IgA is an antibody to viral, bacterial, or dietary antigens [2–9]. Thus the antibody is probably produced as part of the specific host immune response to various environmental antigens. Such reports strengthen the possibility of a relationship between mucosal immunity and the pathogenesis of IgAN. Nevertheless, attempts to isolate a specific IgA-circulating immune complex-associated antigen in patients with IgAN have been unsuccessful.

We have showed that such mucosal infections as pharyngitis are often associated with the acute onset of IgAN [10]. Then IgAN is an immune complex disease that is caused by a poor mucosal immune response to environmental antigens to which the patient has been chronically exposed. We previously observed that *Haemophilus parainfluenzae* (HP) is more commonly isolated from the pharynx of patients with IgAN than from those with other diseases [11]. We have also identified the glomerular deposition of outer membranes of HP antigens (OMHP) and an increased serum concentration of IgA antibodies against OMHP in patients with IgAN [11].

The present study investigated the relationship among IgA, IgG, and IgM antibodies against OMHP in the sera of patients with IgAN.

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PATIENTS AND METHODS

IgAN was diagnosed in 44 Japanese patients, 24 males and 20 females, aged 18–56 years (average 32·9 years). For comparison, we evaluated 62 patients with other glomerular diseases (OGD), 33 males and 29 females, whose ages ranged from 8 to 59 years (average 35·9 years). OGD consisted of non-IgA mesangial proliferative GN (non-IgA GN; $n = 24$), membranous nephropathy ($n = 10$), systemic lupus erythematosus (SLE; $n = 9$), membranoproliferative GN ($n = 8$), focal segmental sclerosis ($n = 3$),

diabetic nephropathy ($n = 3$), crescentic GN without anti-neutrophil cytoplasmic antibody ($n = 2$), minimal change nephrotic syndrome ($n = 2$), and thin basement membrane disease ($n = 1$). The 62 patients with OGD were divided into two groups according to the presence or absence of mesangial IgA deposition: group A, patients having predominantly IgG or IgM deposits, together with lesser amounts of IgA deposits in the mesangium ($n = 23$); and group B, patients without IgA deposits in the mesangium ($n = 39$). There was no significant difference in the average value of glomerular filtration rate among patients with IgAN ($92{\cdot}4 \pm 28$ ml/min), group A ($90{\cdot}8 \pm 26$ ml/min), and group B ($93{\cdot}7 \pm 36$ ml/min).

Renal biopsy specimens were examined microscopically in detail using light, immunofluorescence and electron microscopy. The diagnosis of IgAN was based on the following criteria: (i) proliferation of mesangial cells and expansion of mesangial matrix, as determined by light microscopy; (ii) presence of predominant IgA deposits mainly in the mesangium, and occasionally along some peripheral capillary loops in a granular pattern, as determined by immunofluorescence microscopy; and (iii) presence of mesangial electron-dense deposits, as determined by electron microscopy. Diagnoses of SLE, Henoch–Schönlein purpura, and liver cirrhosis were excluded by use of a detailed clinical history, examination, and negative laboratory tests, such as anti-DNA antibody, hypocomplementaemia, or hepatitis B surface antigen.

Blood samples were taken at the time of renal biopsy for measurement of serum levels of IgA, IgG, and IgM. Serum samples were stored at -80°C until use.

Antigens were prepared from a strain of HP isolated from the pharynx of a normal individual and were cultured to the stationary phase in brain–heart infusion broth. A sonicate of HP antigens (SHP) prepared by use of 30-min high-power ultrasound pulses was stored at -80°C in PBS pH 7·4 at a protein concentration of 3·1 mg/ml. OMHP were isolated as described previously [12], with 2% (w/v) sodium N-lauroyl sarcosine used to remove cytoplasmic membrane from SHP.

For the production of antiserum against OMHP, OMHP was injected with Freund's complete adjuvant (FCA) into the footpads of rabbits. Repeated injections were administered during weeks 2 and 4. In week 5, whole blood was obtained and serum was separated. The IgG fraction isolated from rabbit serum was shown to have specificity for OMHP by Ouchterlony double immunodiffusion in agarose gel. No lines of reactivity were demonstrated between the IgG fraction and sonicated *Haemophilus influenzae* (HI) (ATCC 9795), *Pseudomonas aeruginosa* (ATCC 27578 and ATCC 27589), or *Escherichia coli* (JM 109).

Western blot analysis of OMHP utilized electrophoresis of 5-μl samples on one-dimensional SDS–polyacrylamide gradient gels (5–20% polyacrylamide) [13]. Following electrophoresis, protein bands were transferred to a nitrocellulose membrane. Nitrocellulose strips were blocked with 0·2% powdered milk in 0·05% PBS–Tween 20 (PBS-T). After further washing, strips were incubated for 60 min with a 1:1000 dilution in PBS of rabbit antiserum against OMHP, then were washed and incubated for 60 min with peroxidase-conjugated protein A (Zymed Labs, South San Francisco, CA) at room temperature, and finally were washed and stained with diaminobenzidine in the presence of 0·2% H_2O_2. Alternatively, nitrocellulose strips were incubated with a 1:1000 dilution in PBS of serum samples from patients with IgAN and OGD, followed by incubation with peroxidase-conjugated rabbit IgG against human IgA, IgG, or IgM (Dako Japan, Kyoto, Japan) and stained.

To determine whether rabbit antiserum against OHMP could react with another bacterial component, sonicated HI (ATCC 9795), *Ps. aeruginosa* (ATCC 27578 and ATCC 27589), or *E. coli* (JM 109) was also separated by SDS–PAGE, incubated with rabbit antiserum against OMHP and washed, then incubated with peroxidase-conjugated swine anti-rabbit immunoglobulin antiserum (Dako), and washed and stained as described above.

In preparation for immunofluorescence microscopy, renal biopsy specimens were embedded in OCT medium (Miles Labs, Elkhart, IN) immediately after sampling and frozen in an acetone–dry ice mixture. Frozen sections were cut serially at 2–3 μm in a cryostat and stored at -80°C until use. Cryostat sections were fixed in absolute acetone for 10 min and then rinsed in PBS for 15 min. Sections were incubated overnight in moist chambers at 4°C with rabbit antiserum against OMHP or with non-immune rabbit serum, washed in PBS, and incubated with FITC-labelled swine antiserum against rabbit immunoglobulins (Dako) for 30 min. After further washing, sections were incubated with tetramethyl rhodamine isothiocyanate (TRITC)-labelled sheep antiserum against human IgA (Nordic Labs, Tilburg, The Netherlands) for 60 min. Finally, the stained sections were examined with a fluorescence photomicroscope. In addition, absorption experiments were done by replacing the primary antiserum with rabbit antiserum against OMHP that had previously been absorbed with OMHP in all patients positive for OMHP.

ELISA was done according to the modified method of Borradori *et al.* [14]. Briefly, each well of a 96-well polystyrene microtitre plate was coated with 100 μl of OMHP at a final protein concentration of 1·01 μg/ml in carbonate buffer, 0·05 mol/*l* pH 9·5. After incubation overnight at 4°C, wells were washed three times with PBS–T and shaken dry. Unoccupied absorption sites in the wells were blocked by overnight incubation at 4°C with PBS–T containing 0·5% (w/v) bovine serum albumin (BSA). A volume of 100 μl of patient serum diluted 1:1000 with PBS–T was added to the wells of the microtitre plates and incubated for 60 min at 37°C. Plates were then washed three times with PBS–T, 100 μl of a peroxidase-conjugated rabbit IgG against human IgA, IgG, or IgM diluted 1:1000 with PBS–T were added to each well and, after incubation for 60 min at 37°C, the wells were washed three times. A volume of 100 μl of *o*-phenylenediamine at a concentration of 33 mg/ml and 0·018% (w/v) H_2O_2 in a phosphate-citrate buffer, 0·1 mol/*l* pH 4·9 were added to each well. After incubation at room temperature for 30 min in the dark, the reaction was stopped by the addition of 100 μl of H_2SO_4, 0·75 mol/*l*. Absorbance was then read at 492 nm.

For amino-terminal amino acid sequence analysis, OMHP was separated by SDS–PAGE under the same conditions as already described. After the SDS–PAGE, protein bands were transferred to a PVDF membrane (BioRad Labs, Hercules, CA). The amino-terminal sequence of the components of OHMP recognized by both rabbit antiserum against OMHP and IgAN patient sera was determined by automated Edman degradation in an Applied Biosystem 470-A gas-phase sequencer using transferred membranes.

Statistical analysis

Numerical data are expressed as mean $\pm$ s.d. Differences were tested for significance by the Mann–Whitney *U*-test. Proportions were compared by χ^2 testing and by Fisher's exact probability test,

where appropriate. Simple linear correlation and Spearman's test were used to evaluate correlations between variables. A level of $P < 0{\cdot}05$ was accepted as statistically significant.

RESULTS

Histopathological findings

Renal biopsy specimens of all 106 patients studied exhibited variable degrees of mesangial hypercellularity, an increase in mesangial matrix, glomerular sclerosis, and tubulo-interstitial changes, depending on the duration and severity of the renal disorder. Immunofluorescence microscopy demonstrated mesangial IgA deposition in all 44 patients with IgAN; IgG was present in 26 patients, and IgM in 22 patients. Of 62 patients with OGD, 23 had mesangial deposition of IgA (group A); IgG was present in 19 patients, and IgM was present in 15 patients. No glomerular IgA deposits were observed in 39 patients with OGD (group B); IgG was present in 17 patients, and IgM in 14 patients.

Components of OMHP recognized by rabbit antiserum and by patient sera

As observed by immunoblotting, the rabbit antiserum against OMHP recognized four components of OMHP, having molecular weights of 19·5, 30, 33, and 40·5 kD, as previously reported [11]. Immunoblotting showed that IgA antibodies against OMHP in the sera from patients with IgAN bound to the same four components of OMHP as did rabbit antiserum against OMHP (Fig. 1). IgA antibodies against OMHP binding to the identical components of OMHP were observed in the sera of patients with OGD. IgG and IgM antibodies against OMHP binding to these components were also found in the sera of patients with IgAN and OGD. Rabbit antiserum against OMHP failed to recognize any component of *Ps. aeruginosa* or of *E. coli*, except for one component of HI of molecular weight 18·5 kD.

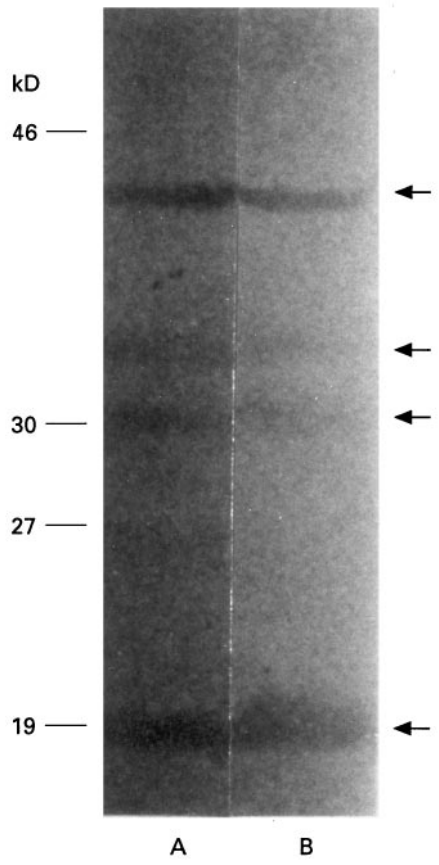

Fig. 1. Immunoblotting of SDS–PAGE gels of rabbit antiserum against outer membranes of *Haemophilus parainfluenzae* antigens (OMHP) and the serum of an IgA nephropathy patient. Rabbit antiserum against OMHP reacted with four components of OMHP with molecular weights of 19·5, 30, 33, and 40·5 kD (lane A). The serum of a patient with IgA nephropathy also recognized the same component of OMHP (lane B).

Amino-terminal amino acid sequence analysis

An amino-terminal amino acid sequence of a 19·5 kD band of OMHP could be determined as VSTVSYGEE. A computer search for sequence similarity in Gen Bank showed that the sequence was 77·8% identical with the sequence from residues 123 to 131 of outer membrane protein (OMP) P6 precursor of HI [15], as shown in Fig. 2. An amino-terminal amino acid sequence of a 30-kD band of OMHP was determined as APQENTFYAG**AGQA. This amino-terminal sequence was 87·5% identical with the sequence from residues 30 to 45 of OMP P5 of HI type b [16]. The sequence of a 40·5-kD band of OMHP was determined as AVVYDNEGTK-VELNG*L*VI, and was 80% identical with the sequence from residues 21 to 40 of OMP P2 (porin) of non-typeable HI [17].

Detection of OMHP in glomeruli

Rabbit antiserum against OMHP bound to the mesangium and partially to the capillary loops of 41 biopsy specimens from the 44 patients with IgAN. A total of 41 of these patients were positive for OMHP in the glomeruli with the same distribution as that of IgA, as previously reported [11]. Three of the 62 patients with OGD (two with non-IgA GN and one with SLE in group A) showed staining with rabbit antiserum against OMHP. In absorption studies, all 44 biopsy specimens positive for OMHP were faint or negative for OMHP. No glomeruli were stained with non-immune rabbit antiserum in patients with IgAN and OGD. The difference between IgAN and OGD in the occurrence of OMHP was highly significant ($P < 0{\cdot}001$).

Serum levels of IgA, IgG, and IgM antibodies against OMHP (Fig 3)

Patients with IgAN showed a significantly higher level of serum IgA antibodies ($P < 0{\cdot}0005$) and IgG antibodies ($P < 0{\cdot}001$) against OMHP than those with group B. Group A showed a significantly higher level of IgA antibodies against OMHP than group B ($P < 0{\cdot}0005$). The levels of IgM antibodies did not differ significantly among group A, group B, and patients with IgAN.

Correlation among IgA, IgG, and IgM antibodies against OMHP

Patients with IgAN showed a positive correlation between IgA and IgG antibodies ($P < 0{\cdot}02$), between IgA and IgM antibodies ($P < 0{\cdot}02$), and between IgG and IgM antibodies ($P < 0{\cdot}05$) against OMHP (Table 1). Groups A and B showed no correlation between IgA and IgG antibodies, between IgA and IgM antibodies, or between IgG and IgM antibodies against OMHP. The serum IgA level was significantly higher in patients with IgAN (387 ± 108 mg/dl) and in group A (364 ± 152 mg/dl) than in group B (253 ± 109 mg/dl) ($P < 0{\cdot}01$). There was no significant difference in the serum IgG level among patients with IgAN

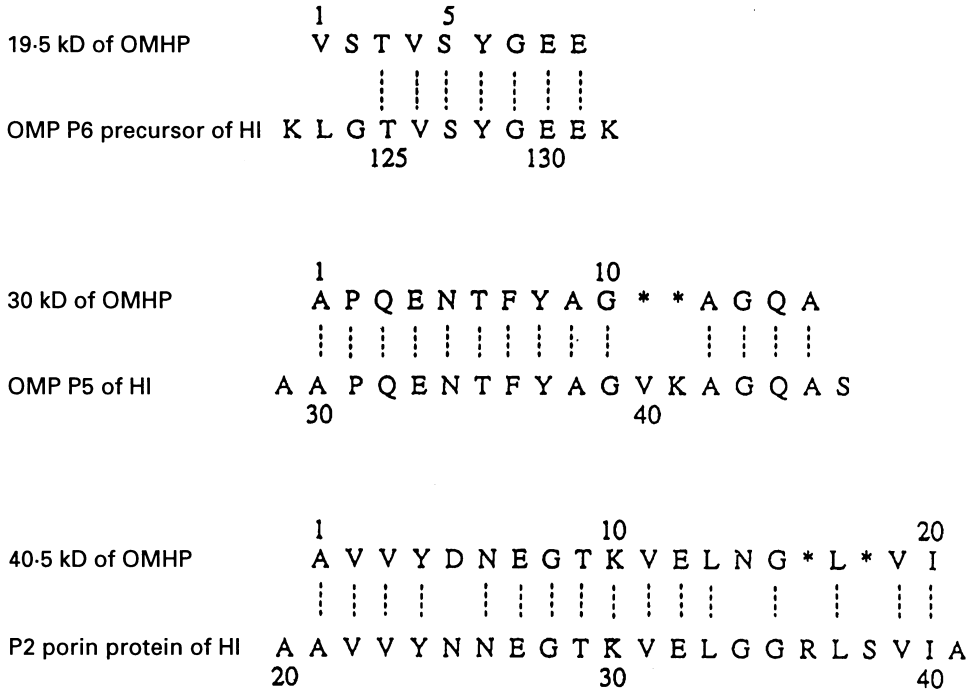

Fig. 2. Amino-terminal acid sequence of three components of outer membrane of *Haemophilus parainfluenzae* antigens (OMHP) recognized by both rabbit antiserum and the sera of IgA nephropathy patients. OMP, Outer membrane protein; HI, *Haemophilus influenzae*. *Not identified.

(1324 ± 368 mg/dl), group A (1351 ± 667 mg/dl), and group B (1305 ± 549 mg/dl). There was no significant difference in the serum IgM level among patients with IgAN (174 ± 106 mg/dl), group A (187 ± 95 mg/dl), and group B (189 ± 113 mg/dl). Patients with IgAN showed no correlation between IgA antibodies against OMHP and the serum IgA level, or between IgG antibodies against OMHP and serum IgG level (Table 2). Groups A and B exhibited positive correlations between IgG antibodies against OMHP and serum IgG level, and between IgM antibodies against OMHP and serum IgM level.

DISCUSSION

Determining the cause of IgAN is very important, as it is regarded as the most common of all glomerular diseases [18,19]. The deposited IgA is likely to be an antibody against environmental antigen such as viral, bacterial, or dietary antigen, although the pathogenesis is not known. Postulations concerning the antigen of IgAN are borne out by studies variously implicating cytomegalovirus antigens [2], Epstein–Barr virus antigens [3], hepatitis B virus antigen [4], herpes simplex virus and adenovirus antigens [5], *E. coli* antigens [6], gluten [7], gliadin [8], and casein and soybean [9]. We speculate that, in the main, IgAN is an immune complex disease that results from an abnormal immune response of the mucosa to environmental antigens to which the patient is chronically exposed. Although these experiments have been repeated by numerous groups, results were not reproducible. We recently reported that HP is involved in the etiology of IgAN [11]. HP is part of the normal flora of the mouth and nasopharynx, and is occasionally found in the intestine. Clinical infection is the result of local or blood stream invasions from these sites, usually dental diseases, other oral trauma, or respiratory tract infection. The present study demonstrated that OMP P6 precursor, OMP P5, and OMP P2 (porin) of OMHP are deposited in the glomeruli of patients with IgAN, and also that IgA, IgG, and IgM classes of antibodies against these OMHP are present in the sera of patients with IgAN. These results suggest that IgA antibodies against OMHP coexists with OMHP as an immune complex in the glomeruli of patients with IgAN. These findings may explain the various combinations of IgG and IgM that accompany the IgA which predominates in the glomeruli of these patients, as observed by numerous researchers.

Several mechanisms are responsible for the increased production of IgA: (i) an increase in IgA-specific helper T cell activity [21]; (ii) hyperactivity of IgA-specific B cells [22]; and (iii) a defect in IgA-specific suppressor T cell activity [23]. This study showed that patients with mesangial IgA deposits, patients with IgAN and group A, have significantly higher serum IgA levels than patients without mesangial IgA deposits. Some researchers report an increase in the production of IgA, IgG, and IgM by unstimulated cultures of peripheral blood mononuclear cells [24]. Our study showed that patients with IgAN have significantly higher levels of IgA and IgG antibodies against OMHP, and that there was no correlation between the serum IgA level and IgA antibodies against OMHP, or between the serum IgG level and IgG antibodies against OMHP. This study also demonstrated that groups A and B show a positive correlation between serum IgA level and IgA antibodies against OMHP or between serum IgG level and IgG antibodies against OHMP. Findings suggest that production of IgG antibodies and, especially, of IgA antibodies against OMHP is increased with IgAN.

It was recently reported that $CD4^+$ T cells with receptors for the Fc portion of IgA specifically enhance the switch of IgM-bearing cells to IgA-bearing cells, which may be responsible for polyclonal activation of IgA production in patients with IgAN [21]. We demonstrated a positive correlation between IgA and IgG

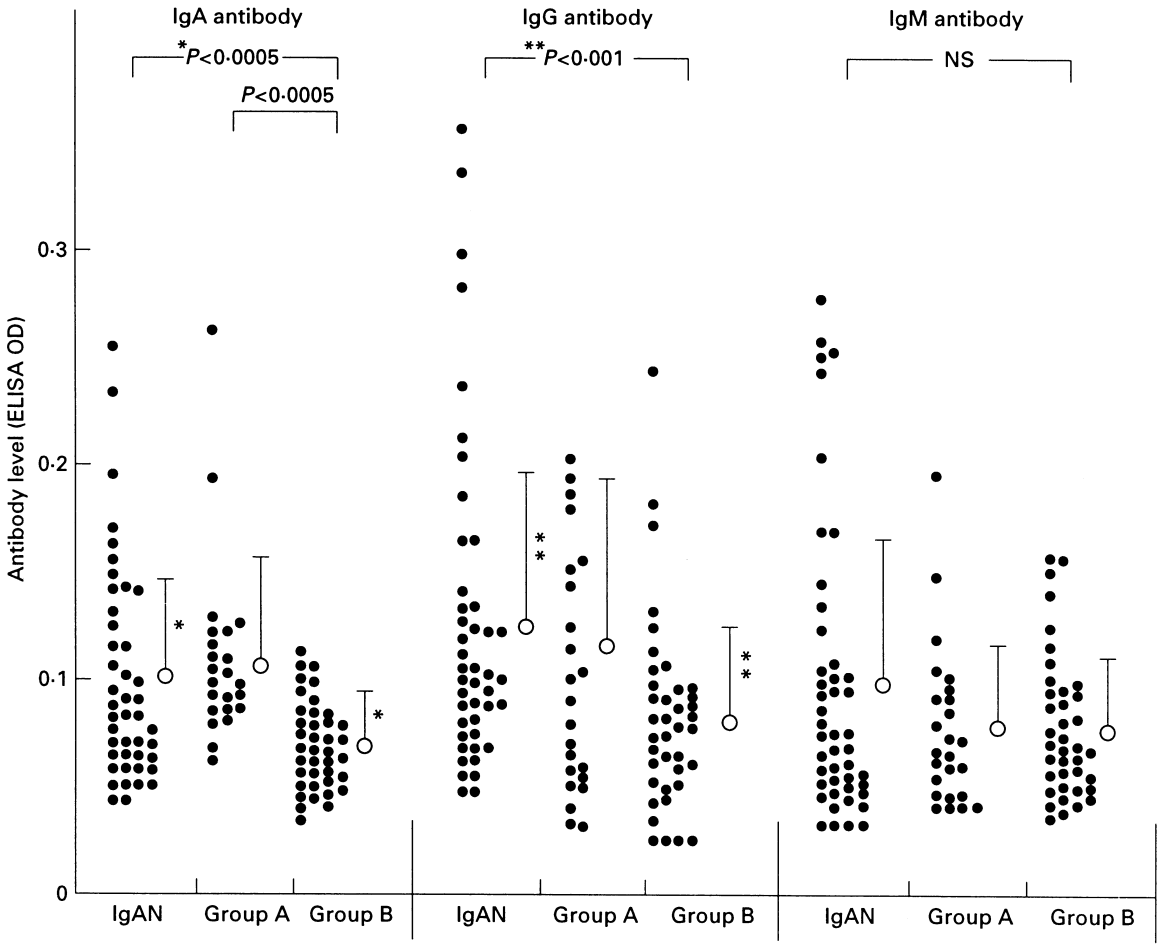

Fig. 3. Serum levels of IgA, IgG, and IgM antibody against outer membranes of *Haemophilus parainfluenzae* antigens (OMHP) (ELISA) in 44 patients with IgA nephropathy (IgAN) and 62 patients with other glomerular diseases consisting of group A and group B. Patients with IgAN showed a significantly higher level of IgA antibody ($P < 0{\cdot}0005$) and IgG antibody ($P < 0{\cdot}001$) against OMHP than did group B patients. OD, Optical density; O, Mean; vertical bar shows 1 s.d.

antibodies, between IgA and IgM antibodies, and between IgG and IgM antibodies against OMHP only in patients with IgAN. These results suggest that patients with IgAN have a polyclonal activation against OMHP, with switching of production from one isotype to another; from IgM to IgA.

Table 1. Correlations among IgA, IgG, and IgM antibodies against outer membranes of *Haemophilus parainfluenzae* antigens in patients with IgA nephropathy (IgAN) and groups A and B

	IgA antibodies: IgG antibodies	IgA antibodies: IgM antibodies	IgG antibodies: IgM antibodies
IgAN	0·345*	0·340*	0·328*
Group A	0·039	−0·186	−0·199
Group B	0·156	−0·062	−0·070

*$P < 0{\cdot}05$.

Group A, 23 patients having predominantly IgG or IgM deposits together with lesser amounts of IgA deposits in the mesangium. Group B, 39 patients having predominantly IgG or IgM deposits withoug IgA deposits in the mesangium

In conclusion, these findings suggest that OMP P6 precursor, OMP P5, or OMP P2 (porin) of HP and antibodies, especially IgA antibodies against these OMHP, coexist in the glomeruli of patients with IgAN, and that production of antibodies against these OMHP increases with switching of production from one

Table 2. Correlations between antibodies against outer membranes of *Haemophilus parainfluenzae* antigens and serum immunoglobulin level in patients with IgA nephropathy (IgAN) and groups A and B

	IgA antibodies: S-IgA	IgG antibodies: S-IgG	IgM antibodies: S-IgM
IgAN	0·289	0·240	0·669*
Group A	0·235	0·553****	0·638**
Group B	0·279	0·561***	0·478***

S, Serum

*$P < 0{\cdot}0001$; **$P < 0{\cdot}001$; ***$P < 0{\cdot}005$; ****$P < 0{\cdot}01$.

Group A, 23 patients having predominantly IgG or IgM deposits together with lesser amounts of IgA deposits in the mesangium. Group B, 39 patients having predominantly IgG or IgM deposits without IgA deposits in the mesangium.

isotype to another in patients with IgAN. OMHP is suggested to have a role in the pathogenesis of IgAN.

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