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Abnormalities of the IgA immune system in members of unrelated pedigrees from patients with IgA nephropathy

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SUMMARY

In the last few years many investigators have reported the recurrence of primary IgA nephropathy (IgAN) or the presence of persistent microhaematuria and/or proteinuria in family members of patients with IgAN. Our study was undertaken to investigate the relevance of abnormalities in the regulation of the IgA and IgM immune system in microhaematuric and asymptomatic family members of IgAN patients. Fifty-four out of 120 members of nine unrelated pedigrees were examined by urinalysis; polymeric IgA (pIgA), IgA rheumatoid factor (IgARF), IgA1-IgG immune complexes (IgA1-IgG IC) and IgA1-IgM IC, and other immunoglobulins were measured in serum samples. Moreover, we studied the production of immunoglobulins, pIgA and IgARF by peripheral blood mononuclear cells (PBMC) in basal conditions and after pokeweed mitogen (PWM) stimulation. Our data demonstrate that persistent microhaematuria was present in 24% of relatives. High serum levels of IgA, mainly pIgA and IgARF, IgA1-IgG IC and IgA1-IgM IC occurred in 66% of relatives. Abnormal spontaneous production of IgA by PBMC and after PWM stimulation was present in 64% of family members. Interestingly, high serum levels of IgM and abnormal production of this immunoglobulin by PBMC were observed in relatives. However, the immunological abnormalities did not correlate in any way with the presence of urinary abnormalities such as microhaematuria, which was most likely determined by an underlying glomerular alteration.

Keywords IgA nephropathy immunoglobulin A relatives

INTRODUCTION

High serum levels of IgA, mainly polymeric IgA (pIgA), circulating IgA immune complexes (IC) and IgA rheumatoid factor (RF) are frequent findings in patients with IgA nephropathy (IgAN) [1]. Furthermore, there is evidence for increased *in vitro* production of IgA by peripheral blood mononuclear cells (PBMC) and overproduction of IL-2 [2–4].

Although systematic screening urinalysis testing in family members of patients with glomerulonephritis is not a common practice for nephrologists, in the last few years many investigators have reported the occurrence of IgAN or the presence of persistent microscopic haematuria and/or proteinuria in family members of patients with this disease [5–9]. Therefore, two forms of disease have been identified as follows: (i) familial IgAN, characterized by the presence of the disease in siblings or other first-degree relatives; (ii) sporadic IgAN, occurring only in one member of the family [10].

Several studies have described various immune abnormalities involving the regulation of IgA synthesis in relatives of IgAN patients [5–7,10–12]. To our knowledge, no extensive studies have been performed concerning the production of pIgA and IgA RF by PBMC in relatives of IgAN patients. Thus, this study was undertaken to investigate this aspect and to assess whether serum and *in vitro* IgA abnormalities might be related to the development of disease in asymptomatic family members of IgAN patients.

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PATIENTS AND METHODS

IgAN patients

Thirty-eight (29 male, nine female) IgAN patients were diagnosed according to the following criteria: (i) no evidence of systemic lupus erythematosus, Schönlein–Henoch purpura or hepatic disease; (ii) recurrent episodes of macrohaematuria concomitant with upper respiratory tract infections or persistent microhaematuria; (iii) mesangial deposits of IgA in glomeruli. Their age distribution ranged from 19 to 50 years (mean 35·7 years). Nine families of 11 IgAN patients participated in the study. Four out of the 11 IgAN patients were considered as having familial IgAN (two sibling pairs). Of the remaining, six IgAN patients had at least one relative with persistent glomerular microhaematuria (suspected diagnosis of familial IgAN), while only one patient did not have any family member with urinary abnormality.

Family members

Fifty-four out of 120 first-degree relatives of the 11 IgAN patients, belonging to nine unrelated pedigrees, were examined. They were 13 parents, 17 siblings and 24 sons of IgAN patients. The age of the family members ranged from 5 to 77 years (mean 41·6 years). Sex distribution was 23 males and 31 females. Seventeen subjects were aged under 18 years, 29 from 18 to 60 years and eight individuals were aged above 60 years.

Controls

Staff members and blood donors, matched for age and sex with IgAN patients and their relatives, represented normal controls. Neither patients, nor relatives or controls, had taken corticosteroids or immunosuppressive drugs before the study.

Appropriate informed consent was obtained from patients and family members.

Serum sample collection

Blood samples were allowed to clot in a sterile pyrogen-free tube (Vacutainer, Becton Dickinson, Rutherford, NJ) at 37°C for 2 h and then centrifuged at 2000 ***g*** at room temperature for 20 min. Serum samples were stored in small aliquots at −70°C until tested. All serological studies were performed in double blind trials.

Serum levels of IgG, IgA and IgM were determined by radial immunodiffusion technique using Nor-partigen plates (Behringwerke AG, Marburg, Germany). Mean values obtained from serum samples of 56 healthy blood donors were expressed as mg/dl. They were 1080·7 ± 211·2 (mean ± s.d.) for IgG, 188·3 ± 69·4 for IgA and 143·6 ± 69·4 for IgM.

Circulating IgA1-IgG and IgA1-IgM IC were measured by solid phase Jacalin ELISA, described elsewhere [13], in serum samples previously treated with 1 ml of polyethylene glycol 6000 (Merck, Munich, Germany). Data were expressed as optical density (OD). All specimens were assayed the same day. The 95th percentile obtained from serum results of 33 healthy blood donors was assumed to be normal range. It was < 0·25 OD for IgA1-IgG IC and < 0·56 OD for IgA1-IgM IC.

PBMC isolation

Mononuclear cells were isolated from heparinized blood by Boyum's method [14]. The cell suspension recovered at the interface was resuspended in RPMI 1640 (Eurobio, Paris, France) and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM) and 10% heat-inactivated fetal calf serum (FCS). PBMC at a concentration of 2×10^6 cells/ml were stimulated with pokeweed mitogen (PWM), 5 μg/ml for 7 days at 37°C in 5% CO_2, 95% air atmosphere. At the end of this period, cell-free supernatant was obtained by centrifugation at 2000 ***g*** for 10 min and frozen at −20°C until assayed for IgG, IgM and IgA. Immunoglobulin classes were evaluated by ELISA using polyclonal antisera (KPL, Gaithersburg, MD). Immunoglobulin reference standards (Sigma, St Louis, MO) were used to generate the calibration curve.

Detection of polymeric IgA

pIgA were detected by the secretory component solid phase assay [15]. Secretory component was purified from human whey by affinity chromatography. Briefly, human monoclonal IgM were covalently coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden). IgM-Sepharose was incubated for 2 h at 4°C with diluted whey, after which the mixture was poured into a chromatographic column. Absorbed protein was eluted with 1·0 M potassium thiocyanate in 0·01 M potassium phosphate, pH 7·0, dialysed against PBS, and gel filtered on Sephacryl S200 (Pharmacia). Two peaks were obtained, the second containing pure secretory component, as judged by immunoelectrophoresis and double immunodiffusion, using anti-whole human milk (Nordic Immunology, Tilburg, The Netherlands) and anti-secretory component (Dakopatts, Copenhagen, Denmark) antisera.

Microtitre plates (Dynatech Deutschland GmbH, Plochingen, Germany) were coated overnight at 4°C with 6 μg/ml solution of purified free secretory component in 100 μl of PBS followed by PBS containing 0·2% human serum albumin (HSA; Sigma) and 0·2% gelatin (Difco Laboratories, Detroit, MI). Individual column fractions or samples were added to the wells and incubated overnight at 4°C. After three washes with PBS-Tween, bound pIgA were measured by addition of peroxidase-conjugated $F(ab')_2$ fragment of goat anti-human IgA followed by four more washes with the specific substrate. Thirty minutes later, the colour reaction was measured by a Titertek Multiskan (Flow Laboratories, Rockville, MD) at 405 nm. The results were expressed as OD readings. The mean value of serum pIgA in healthy subjects was 0·22 ± 0·09 (mean ± s.d.).

Detection of IgA rheumatoid factor

IgA RF was detected by ELISA as described previously [15,16] with minor modifications. Microplate wells (Dynatech) were coated overnight at 4°C with a 10 μg/ml solution of normal human IgG in 100 μl of PBS. After washing with PBS-Tween and blocking with PBS containing 0·2% HSA and 0·2% gelatin, 100 μl of either standards or samples were added, and the plates were incubated in a moist chamber at room temperature for 18–20 h. The standards used were multimeric IgA RF purified from the serum of a patient with IgAk-IgG essential mixed cryoglobulinaemia by gel chromatography at acid pH. Plates were washed three times in PBS-Tween, and 100 μl of peroxidase-conjugated $F(ab')_2$ fragment goat anti-human serum IgA (Cappel Labs, Downingtown, PA), diluted 1:2000 in PBS-Tween-HSA 0·1%, were added to the wells. After incubation for 1 h at 37°C, the wells were washed four times, and 100 μl of the substrate were added (ABTS-Hydrogen Peroxide, Kirkegaard and Perry Lab. Inc. Gaithersburg, MD). After incubation for 30 min at room temperature, the absorbance of each well at 405 nm was read with a Titertek Multiskan (Flow) interfaced to an Apple IIE computer to automate the estimations. The results were expressed as μg/ml. The mean value of IgA RF in healthy subjects was 6·9 ± 4·2 (mean ± s.d.).

Urinanalysis

Microhaematuria was detected by N-Multistix (Ames) on urine specimens three consecutive times in all family members of IgAN patients at the time of the study. Furthermore, specimens from subjects with microhaematuria were analysed by Addis count and by phase-contrast microscopy. Microhaematuric subjects were defined as having urinary erythrocyte excretion of more than 1000 cells/min and glomerular haematuria.

Statistical analysis

Values were expressed as means ± s.d. Data were compared by Wilcoxon test (for unpaired data) and Mann–Whitney *U*-test

Table 1. Families of IgA nephropathy (IgAN) patients

Family no.	IgAN patients	No. of relatives with microhaematuria	Serum study: No. of members studied	Serum study: Per cent of relatives with serum IgA abnormalities	*In vitro* study: No. of members studied	*In vitro* study: Per cent of relatives with abnormal IgA synthesis
1	2	1	6/17	83	5/17	60
2	1	4	6/9	83	3/9	33
3	2	1	5/20	80	4/20	75
4	1	1	11/25	73	8/25	87
5	1	2	4/13	25	4/13	75
6	1	1	8/10	62	3/10	66
7	1	3	6/11	50	4/11	75
8	1	0	4/6	75	4/6	75
9	1	2	4/9	75	4/9	0
Total	11	13	54/120		39/120	

(for paired data). Percentages were compared by the χ^2 test with Yates's correction (for small numbers). Statistical comparison among different groups was performed by ANOVA.

RESULTS

Clinical presentation

Table 1 shows the nine unrelated pedigrees of the IgAN patients. Fifty-four relatives out of 120 individuals participated in the study. They were distributed into three generations. Urinalysis revealed persistent microhaematuria in 13/54 relatives (24%). Family no. 1 and no. 3 had two members with IgAN. In family no. 2 two microhaematuric sons were twins; in the same family two more relatives revealed persistent microhaematuria. In three families at least two members had microhaematuria. In family no. 8 no urinary abnormalities were observed in relatives. Ten IgAN patients had serum creatinine less than 1·5 mg/dl, while one had end-stage kidney disease and received periodic haemodialytic treatment. All relatives had normal serum creatinine.

Familial distribution of immunological IgA abnormalities

Thirty-six out of 54 relatives (66%) had at least one serum IgA abnormality and 15/54 (27%) relatives showed more than one. In six of the nine families studied, over 70% of relatives studied showed abnormalities of serum IgA (Table 1). Moreover, serum IgA abnormalities were present in nine out of 13 family members (69%) with persistent microhaematuria.

Fifty-three percent of relatives younger than 18 years showed serum IgA abnormalities; the frequency was 69% in family members aged between 19 and 40 years and 85% in those more than 40 years and less than 60 years old. In older relatives (>60 years), 62% had serum IgA abnormalities.

In vitro IgA synthesis by PBMC was investigated in 39 subjects, since large amounts of blood samples from children were not available. Increased production of IgA, pIgA and IgA RF in both basal conditions or PWM stimulation was found in 33–87% of relatives of eight unrelated pedigrees, while no *in vitro* IgA abnormalities were observed in one family (Table 1).

Percentages of serum and *in vitro* IgA abnormalities in family members distributed according to age were not statistically significant, while a statistically significant difference ($P<0·05$) was found for the *in vitro* IgA abnormalities between relatives younger than 18 years old and the other groups.

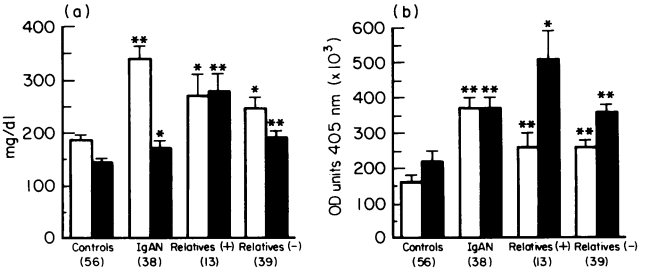

Fig. 1. Serum levels of (a) IgA (□) and IgM (■), (b) IgA1-IgG (□) and IgA1-IgM immune complexes (■) in controls, IgAN patients and relatives with persistent microhaematuria (relatives+) and those with normal urinalysis (relatives−). Values are mean±s.e.m. $*P<0·05$; $**P<0·001$.

Serum IgA and circulating IgA1 IC

Serum levels of IgA were significantly higher in IgAN patients ($P<0·0001$) and in their relatives either with microhaematuria ($P<0·04$) or without urinary abnormalities ($P<0·02$) than in controls (Fig. 1a). High significant values of IgM were also found in IgAN patients ($P<0·04$) and in relatives with microhaematuria ($P<0·0007$) or with no urinary abnormalities ($P<0·006$) (Fig. 1b). Serum levels of IgG were not significantly increased (data not shown).

Twenty-eight out of 53 relatives (53%) had increased serum levels of IgA1-IgG IC and 8/53 had high values of IgA1-IgM IC. Mean values of IgA1-IgG IC and IgA1-IgM IC were significantly higher in IgAN patients ($P<0·000001$; $P<0·0007$), in relatives with microhaematuria ($P<0·003$; $P<0·02$) or without urinary abnormalities ($P<0·0001$; $P<0·008$) than controls (Fig. 1b).

Serum polymeric IgA and IgA rheumatoid factor

Serum levels of pIgA were increased in IgAN patients ($P<0·001$) and in 13/50 relatives (26%), of whom four revealed persistent microhaematuria (Fig. 2a).

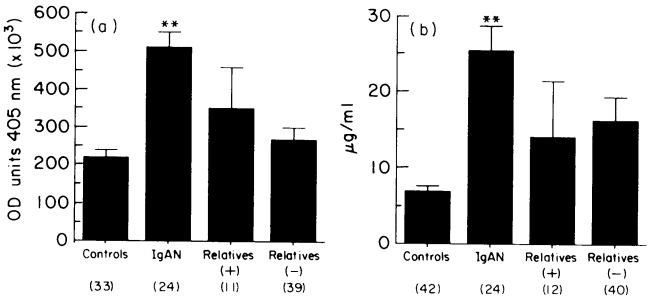

Fig. 2. Serum levels of polymeric IgA (a) and IgA rheumatoid factor (b). Values are mean ± s.e.m. $*P < 0.05$; $**P < 0.001$.

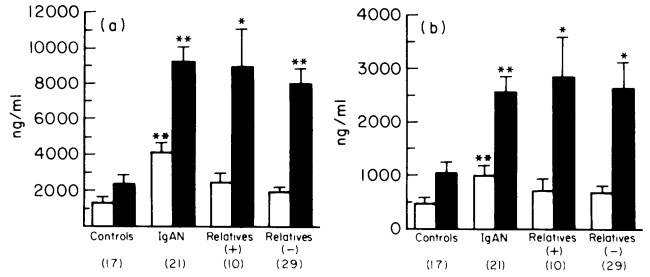

Fig. 3. The *in vitro* production of IgA (a) and IgM (b). Values are mean ± s.e.m. $*P < 0.05$; $**P < 0.001$. □, Spontaneous; ■, pokeweed mitogen.

High values of IgA RF were found in IgAN patients ($P < 0.005$) (Fig. 2b) and in 13/52 relatives (25%), of whom two had persistent microhaematuria. However, we did not find any significant difference between normals and relatives, since the values showed large variability. IgA RF was persistently present in five family members of two IgAN patients.

Immunoglobulin synthesis by PBMC

Increased spontaneous production of IgA by cultured PBMC was present in IgAN patients ($P < 0.006$), while no differences were found in relatives with microhaematuria and in those with normal urine. The production of IgA increased mainly after PWM stimulation of PBMC both in patients ($P < 0.008$) and in relatives with urinary abnormalities ($P < 0.02$) and in those with normal urine ($P < 0.001$) (Fig. 3a).

IgM values in PBMC supernatants of IgAN patients were significantly higher than controls both in basal conditions ($P < 0.006$) and after PWM stimulation ($P < 0.001$). Significantly increased production of IgM from PBMC stimulated with PWM was observed in relatives with urinary abnormalities ($P < 0.05$) and in those with normal urine ($P < 0.02$) (Fig. 3b). No significant variation of IgG production was observed in patients and relatives after PWM stimulation (data not shown).

Polymeric IgA and IgA rheumatoid factor synthesis by PBMC

Spontaneous production of pIgA was significantly higher in IgAN patients than in controls ($P < 0.01$). By contrast, no effect of PWM stimulation was observed in relatives (Fig. 4a). Spontaneous production of IgA RF was significantly increased in IgAN patients ($P < 0.008$) and in normal relatives ($P < 0.001$). This production increased but not significantly after PWM stimulation in patients ($P < 0.07$) and their normal relatives

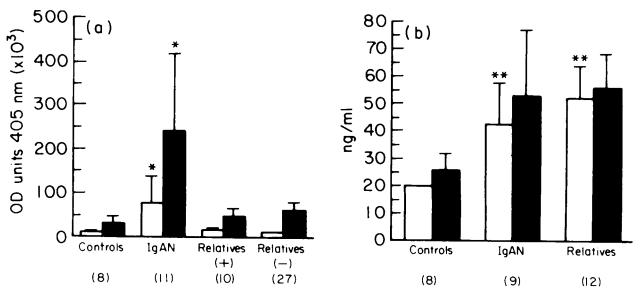

Fig. 4. The *in vitro* production of polymeric IgA (a) and IgA rheumatoid factor (b). Values are mean ± s.e.m. $*P < 0.05$; $**P < 0.001$. □, Spontaneous; ■, pokeweed mitogen.

($P < 0.08$) (Fig. 4b). Relatives with persistent microhaematuria were not studied.

DISCUSSION

The true incidence of familial IgAN is unknown because few renal units systematically monitor the urine of family members and perform renal biopsy in those subjects with persistent microhaematuria. To our knowledge, no extensive familial studies have been done concerning IgA production by PBMC in culture. The present study showed that persistent microhaematuria appeared in 24% of apparently healthy family members of IgAN patients. In addition, one or more serum immunological abnormalities of the IgA system were present in 66% of relatives of nine unrelated pedigrees from 11 IgAN patients. Furthermore, spontaneous and PWM-induced abnormal production of IgA was present in 64% of family members independently of urinary abnormalities.

Increased serum IgA levels in healthy family members have been observed by many investigators [11,17–20]. Our data confirm the high frequency of increased serum IgA values in relatives except for paediatric subjects. However, it is very interesting to note that high serum levels of IgM and abnormal spontaneous production and PWM stimulation of this immunoglobulin by PBMC were present in relatives. We have previously observed an increased basal or phytohaemagglutinin (PHA)-induced synthesis of IL-4 by PBMC from IgAN patients [21]. Moreover, recent data from Yano *et al.* [22] have demonstrated an altered production of IgA induced by IL-4 on PBMC from IgAN patients. This lymphokine controls the *in vivo* immunoglobulin isotype selection [23]. Thus, we can hypothesize that this lymphokine could be involved in the abnormalities of IgA, and perhaps IgM, synthesis by PBMC of IgAN relatives.

High serum values of pIgA were observed in IgAN patients and in 26% of family members; microhaematuria was not a discriminant parameter for this abnormality. The physiological role of serum pIgA is not clear, but there is strong evidence for its implication in IgAN since it is increased in 67% of routinely investigated patients [24] and is present in the mesangial deposits of renal biopsies [25]. Delacroix *et al.* [26] and Jones *et al.* [27] demonstrated the highest proportion of pIgA in youngsters. The presence of increased pIgA values in family members of IgAN patients may be explained by a preferential production of pIgA following initial antigen exposure, as occurs in the younger age group.

Czerkinsky *et al.* [28] and Sinico *et al.* [29] have reported the occurrence of polymeric, IgA RF suggesting that IgA RF may play an important role in the pathogenesis of the disease [15].

Our data demonstrate that increased IgA RF levels occurred in 25% of family members of IgAN patients. The clinical significance of IgA RF in IgAN is unknown, but these findings provide additional evidence for a general perturbation of the IgA system in relatives of IgAN patients.

Our data show that 53% of relatives had increased serum levels of IgA1-IgG IC. The absence of urinary abnormalities in some family members may weaken the hypothesis that the persistence of circulating immune complexes may cause IgAN throughout an indefinite follow-up period.

Increased spontaneous and/or mitogen-stimulated production of IgA *in vitro* has been observed in relatives and siblings of IgAN patients [7,11,19,30,31]. Our data demonstrate an increased spontaneous production of IgA in relatives independently of the presence of urinary abnormalities; this production increased mainly after PWM stimulation. Interestingly, this abnormal production involved, also, the IgM system. These findings may explain the increased values of serum IgA and IgM in relatives.

An abnormal but not insigificant production of pIgA after PWM stimulation was observed in relatives. Furthermore, PBMC of these apparently healthy subjects produced a significant amount of IgA RF spontaneously and after PWM stimulation. Although no correlation was found between *in vitro* production and serum levels, these data demonstrate that abnormalities of the IgA system are latently present in relatives of IgAN patients. In view of these observations we are following the family members of our patients by urinanalysis, since recently some of them have revealed the appearance of persistent microhaematuria.

Many published reports quoted by Levy [32] described a history of renal disease in family members of IgAN patients, but, since no renal biopsy was performed, it is not possible to conclude that these subjects had IgAN. Some members were affected by IgAN and others by urinary abnormalities. Our study shows that when microhaematuria was consistently documented in one quarter of family members, immunological abnormalities of the IgA system were present in 66% of parent offsprings, twins or siblings. These data suggest a possible genetic basis for an IgA disorder.

Whether IgA disorder is a chance phenomenon or the consequence of common exogenous factors, such as dietary or environmental factors, is open to discussion. Julian *et al.* [6] in a familial study devoted to genealogical investigations concluded that the manifested IgAN in related pedigrees was dependent on one or several of the original settlers. In our study we reported IgA abnormalities in family members of nine non-related pedigrees. Therefore, further systemic studies on a large number of affected siblings are needed by using segregation or linkage analysis. However, the invasive nature of the renal biopsy is the major difficulty in the study of familial IgAN, since this procedure is difficult to apply in relatives with modest urinary abnormalities.

We would like to emphasize that, although immunologic abnormalities are common in relatives of IgAN patients, they do not seem to be associated in a causative fashion with the occurrence of urinary abnormalities (i.e. microhaematura and/or proteinuria). This finding is parallel to the observation made on subjects with overt IgAN and, perhaps, indicates that abnormalities of the IgA system lack specificity for the presence of IgAN.

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