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Characterization of circulating idiotypes containing immune complexes and their presence in the glomerular mesangium in patients with IgA nephropathy

J. GONZÁLEZ-CABRERO, J. EGIDO, F. MAMPASO*, M. C. RIVAS & L. HERNANDO *Fundación Jiménez Díaz, Autonoma University—CSIC and *Hospital Ramón y Cajal, Madrid, Spain*

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SUMMARY

The possible pathogenic role for idiotype-anti-idiotype interactions in kidney diseases has recently been suggested. Since patients with IgA nephropathy often present antibodies against alimentary antigens, like bovine serum albumin (BSA), we isolated an idiotypic antibody with BSA specificity from one of these patients. By means of a specific anti-idiotypic antibody raised in rabbits, we have studied the participation of these idiotypes in circulating and renal deposited immune complexes (IC) in patients with IgA nephropathy. On indirect immunofluorescence, the presence of cross-reactive idiotypes was detected in the glomeruli of 12 out of 42 (28%) patients with IgA nephropathy, but in none of 15 membranous or mesangiocapillary nephritis examined. The staining was located within mesangial and paramesangial areas, with a similar, but less intensive, pattern distribution than IgA. Previous adsorption of rabbit anti-idiotype antibodies on an idiotype-Sepharose column completely abolished that staining. A close relationship was found between the presence of cross-reactive idiotypes on mesangial immunoglobulins and the existence of increased levels of serum idiotypes and idiotype-containing IC. Serum analytical ultracentrifugation showed that circulating IC containing idiotypes have chiefly a large (> 19 S) and intermediate (13 S–19 S) size, while those containing anti-BSA antibodies were only between 7 S–13 S fractions, or absent. Our results suggest that in patients with IgA nephropathy, shared idiotypes participate in the formation of circulating and renal deposited IC. It is possible that the apposition of free anti-idiotype to idiotype already bound to glomeruli, and vice versa, could contribute to increasing the amount and size of mesangial immune deposits, and, therefore, facilitate or perpetuate tissue injury.

Keywords idiotypes IgA nephropathy mesangium immune-complexes

INTRODUCTION

IgA nephropathy is now recognized worldwide as one of the most common nephritides. The constant presence of IgA, and sometimes C3 and/or other immunoglobulins in the glomerular mesangium suggest that this disease is immune complex (IC)-mediated. The composition of these IC is, however, to a large extent not known. The available data suggest that IgA-IC in this disease are heterogeneous (Egido *et al.*, 1984a; Czerkinsky *et al.*, 1986).

IgA nephropathy is a disorder characterized by B lymphocyte hyperactivity resulting in increased IgA and antibody formation against exogenous and endogenous antigens (review in Egido *et al.*, 1984b). We have previously demonstrated that about 50% of these patients have IC formed with antibodies to alimentary antigens (bovine serum albumin BSA or ovalbumin) and that the size and the serum levels of IgA-IC present fluctuations after the ingestion of large amounts of protein (Sancho *et al.*, 1983). Since anti-idiotype antibodies seem to play an important role in the setting of numerous and repeated immunization by the same antigen (Cunningham-Rundles, 1982; Male, 1986), we postulated that these antibodies could participate in the formation *in vivo* of IC, competing with alimentary antigens for binding sites on the idiotypic antibodies.

In a recent report, we have described the isolation of an idiotypic antibody with BSA specificity, and the corresponding autologous anti-idiotypic antibodies from a patient with IgA nephropathy (González-Cabrero *et al.*, 1987). By means of anti-idiotypic antibodies raised in rabbits, we observed the presence of increased levels of shared idiotypes in serum in a large group of genetically unrelated patients with this disease. The close correlation between the presence of IgA-IC and the existence of high levels of serum idiotypes suggested that a portion of circulating IC could consist of idiotype-anti-idiotype complexes (González-Cabrero *et al.*, 1987).

Correspondence: Dr J. Egido, Laboratorio de Nefrología, Fundación Jimeńez Díaz, Avda Reyes Católicos 2, 28040 Madrid, Spain.

In recent years, it has been suggested that idiotypic interactions (i.e. idiotype-anti-idiotype IC) may play a role in the pathogenesis of some glomerular diseases. In a model of chronic serum sickness induced in rabbits, the presence of anti-idiotypic antibodies as a component of the glomerular immune deposits was found (Zanetti & Wilson, 1983). In mice undergoing polyclonal B cell activation, idiotypic specificity T15 and their respective anti-idiotypic determinants were detected at renal level (Goldman *et al.*, 1982). More recently, in patients with lupus erythematosus, cross-reactive anti-DNA antibody idiotypes have been identified on tissue-bound immunoglobulins in renal (Isenberg & Collins, 1985) and skin biopsies (Isenberg *et al.*, 1985).

In this paper, we have examined, by means of specific anti-idiotypic antibodies, as previously characterized (González-Cabrero *et al.*, 1987), the presence of shared idiotypes in the mesangium of patients with IgA nephropathy. Furthermore, the size and composition of circulating idiotype-containing IC was also studied.

MATERIALS AND METHODS

Subjects studied

Forty-two kidney biopsies of adult patients with IgA nephropathy (18 of them also with IgG and/or IgM deposits) and 15 patients with membranous or mesangiocapillary nephritis (three of them also with IgA deposits besides IgG) were studied for the presence of shared idiotypes in the glomerular immune deposits. Thirty biopsies were studied retrospectively from material kept in isopentane not longer than 1 year and twelve from formalin-fixed, paraffin-wax-embedded tissue.

Following an overnight fast, blood samples were taken from 13 of the 42 patients with IgA nephropathy and 23 controls to study the presence of shared idiotypes in serum and in IC. In three patients and three controls the composition of these complexes was also studied. The serum obtained by clotting of blood was stored in aliquots at $-70°C$ until tested.

Clinical and serological features

Serum creatinine, proteinuria and haematuria determinations, and immunofluorescence and light microscopy studies were done by routine techniques. Haematuria was defined as the presence of more than 2×10^6 RBC in 12 h urine collections (Addis count).

Detection of idiotypic determinants in renal biopsies

Idiotypic determinants in renal deposits were detected by indirect immunofluorescence technique or by a two-stage immunoperoxidase method by means of the polyclonal anti-idiotypic antibody previously described (González-Cabrero *et al.*, 1987). Briefly, two rabbits were immunized with anti-BSA idiotypes from a patient with IgA nephropathy. Afterwards, serum gammaglobulin fraction from these animals was extensively adsorbed by affinity chromatography on A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), activated with glutaraldehyde (Cambiaso *et al.*, 1975) and cross-linked to pooled normal human gammaglobulins (Sigma Chemicals Co., St. Louis, Mo.).

Cryostat sections from each renal biopsy were incubated with rabbit anti-idiotypic antibody for 45 min. After washing in 0·15 M phosphate-buffered saline (PBS), pH 7·3, fluorescein-labelled goat anti-rabbit immunoglobulins (Nordic Immunology, Tilburg, The Netherlands), diluted 1:10, was applied for 30 min. Then the slides were washed and mounted in glycerin-PBS. Stained sections were viewed under a Leitz Wetzlar microscope (West Germany) equipped for epifluorescence with appropriate filters.

The indirect immunoperoxidase technique in trypsin-digested paraffin sections was performed following the indications of Dormady & Maclver (1980). Briefly, as first antiserum the anti-idiotypic antibody was used; after 30 min of incubation, swine anti-rabbit immunoglobulins were added and finally the soluble complex peroxidase-anti-peroxidase (Dako, Denmark). As chromogenic substrate 3,3′-diaminbenzidine (DAB) (Fluka AG) was used.

The controls of specificity were the following: (1) In some experiments kidney sections were incubated with heterologous anti-idiotypic antibodies previously adsorbed over an affinity column AH-Sepharose 4B to which idiotype (IgG anti-BSA) had been cross-linked; (2) in other experiments, the primary antiserum (the polyclonal anti-idiotypic antibody) was either omitted or replaced by normal rabbit serum.

Examination of BSA in kidney biopsies

To determine the possible presence of antigen (BSA) at the glomerular level, renal biopsy specimens were treated either with PBS, pH 7·4 or with acetic acid elution buffer, pH 4, for 15 min, washed and incubated for 1 h with anti-BSA antibodies from rabbits, purchased commercially (Nordic). After washing, the slides were stained with a fluorescent goat anti-rabbit antiserum (Nordic) for 45 min, washed, mounted and examined.

Detection of shared idiotypes in serum and immune complexes

Cross-reactive idiotypes were measured by a previously described enzyme-linked immunosorbent assay (ELISA) (González-Cabrero *et al.*, 1987). Briefly, wells of polystyrene microtitre plates were coated either with sera from patients and controls diluted in 0·01 M carbonate-bicarbonate buffer pH 9·6 or polyethylene glycol (PEG)-precipitated IC dissolved with 0·2 M glycine-HCl buffer, pH 2·6. After washing, the polyclonal rabbit anti-idiotypic antibodies were added, and after washing again the last incubation with an alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin.

Serum fractionation by sucrose density gradient ultracentrifugation

Serum samples (200 μl) of three patients with IgA nephropathy and three healthy controls were fractionated by ultracentrifugation performed in a linear density gradient of 5–40% w/v sucrose in 0·15 M Tris-HCl buffer, pH 7·4. Gradients were spun for 16 h at 170 000 *g*, 4°C, using a Beckman Model L-8-70 ultracentrifuge (Beckmann Instruments Inc., Palo Alto, CA,), with an SW 50.1 rotor. A parallel gradient contained IgM (19 S), IgG (7 S) and BSA (4·5 S), which were used as molecular weight markers. Gradient tubes were punctured from the bottom, approximately 140 μl fractions (four drops) collected and 300 μl of PBS-azide added to each one.

The profile distribution of serum shared idiotypes was determined in each gradient fraction by ELISA as commented above, but in this assay, the wells of polystyrene microtitre plates were coated with samples of each gradient tube instead of serum.

IgA and IgG anti-BSA antibody levels were also measured in each gradient fraction as described (González-Cabrero *et al.*, 1987). Briefly, test samples obtained from each fraction were added to polystyrene microtitre plates coated with BSA. After removing the unbound material, an incubation with peroxidase labelled goat anti-human IgA and IgG was allowed to proceed.

Statistical analysis

Results obtained from the assays to measure the presence of shared idiotypes and the levels of anti-BSA antibodies were expressed as previously published (González-Cabrero *et al.*, 1987). The mean of the duplicate value (optical density obtained by ELISA) from the test sera or gradient fractions (Ti) was divided by Ui (upper 95% confidence limit in control sera or control gradient fractions) (Egido *et al.*, 1984a). Values of Ti/Ui > 1 were judged as abnormal. A Chi square with Yates' correction was employed to compare the proportion of subjects with renal and serum idiotypes.

RESULTS

Glomerular localization of shared idiotypic determinants

The presence of cross-reactive idiotypes was detected in the mesangium of 12 out of 42 patients (28%) with IgA nephropathy (Fig. 1 right). Deposits of idiotypic determinants were located within mesangial and paramesangial areas with a similar, but less intensive, pattern distribution than IgA (Fig. 1, left). The demonstration of the idiotypes in kidney biopsies was independent of the existence of IgG simultaneously with IgA. Out of 12 patients in whom idiotypes were found, five had isolated IgA deposits, four associated with IgG, two with IgG and IgM and one with IgM. Clinically, all patients in whom the presence of idiotypes was demonstrated in the mesangium had a haematuria; eight out of 12 had proteinuria also. Only two of these 12 patients presented moderate renal failure (serum creatinine between 2 and 3 mg/dl). No significant differences were found in clinical findings, light microscopy, and immunofluorescence pattern with the remaining patients in whom glomerular idiotype staining could not be demonstrated.

The specificity of the idiotype detection was established by several criteria. The incubation of kidney biopsies with rabbit anti-idiotype antibodies adsorbed on an idiotype-Sepharose column (González-Cabrero *et al.*, 1987) completely abolished the previous staining. The possibility of a non-specific binding was further excluded, because the staining also became negative when the first antiserum (rabbit anti-idiotype antibodies) was omitted or when the kidney sections were incubated instead with a normal rabbit serum. Furthermore, we (unpublished) and others (Sinico *et al.*, 1987) have not observed intraglomerular rheumatoid factor activity when renal sections were incubated with fluoresceinated aggregated human IgG. The existence of these idiotypes in the mesangium also seems specific to patients with IgA nephropathy, since no such deposits were found in the kidney biopsies of 15 patients with membranous or mesangiocapillary glomerulonephritis incubated with the unadsorbed anti-idiotypic antibodies. Finally, though for technical and practical reasons a group of kidney biopsies were studied by immunofluorescence and another by immunoperoxidase, both techniques afford comparable results (Dormady & Maclver, 1980). In fact, three cases were studied simultaneously by both

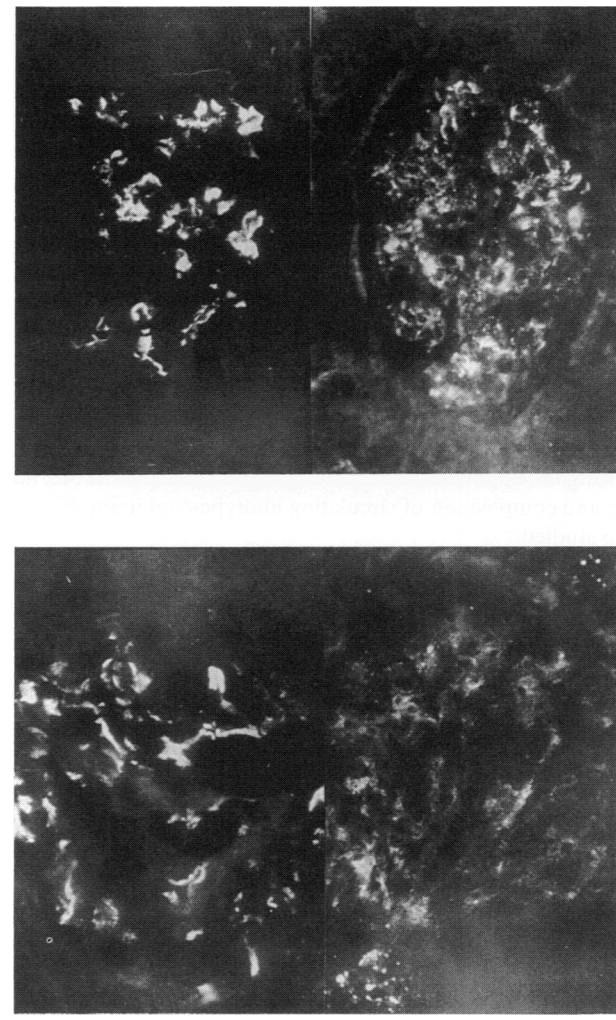

Fig. 1. Glomerular deposits of IgA (left) and idiotypes (right) in two different patients with IgA nephropathy. Note that each picture represents a different glomerulus.

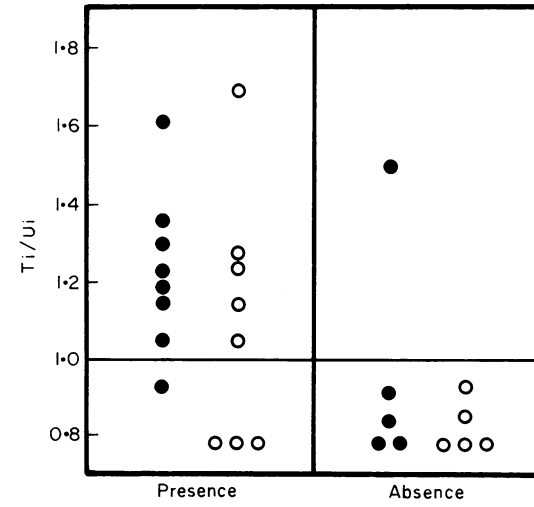

Fig. 2. Relationship between the presence of cross-reactive idiotypes on the mesangial immunoglobulins and the serum levels of idiotypes (●) or idiotype-containing IC (○). Values Ti/Ui greater than 1 are considered positives.

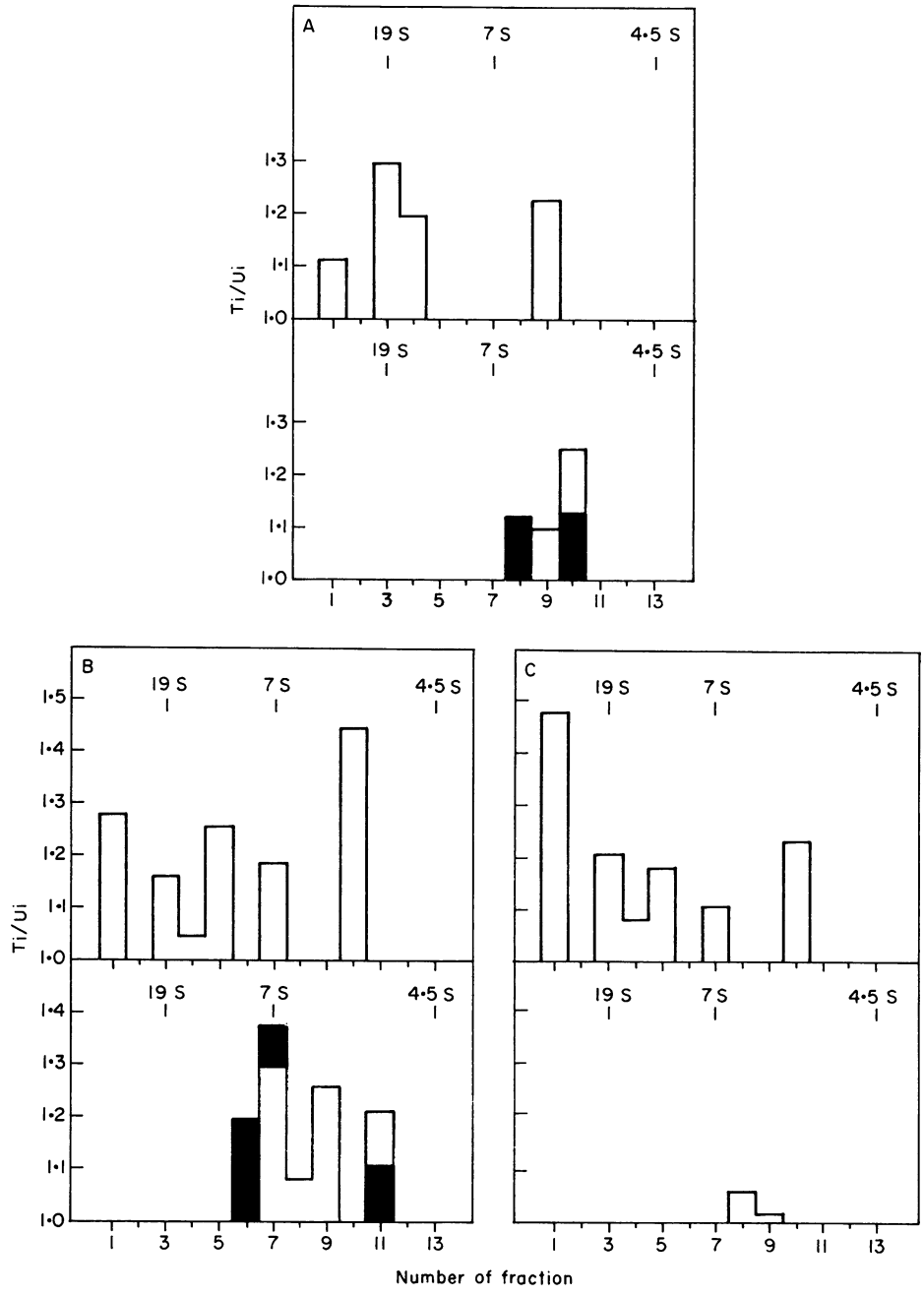

Fig. 3. Serum fractionation by sucrose density gradient ultracentrifugation of three patients (A, B and C) with IgA nephropathy. Upper panels: profile distribution of serum shared idiotypes. Lower panels: profile distribution of anti-BSA antibodies. IgA (■); IgG (□).

methods in a blind manner by the two pathologists involved in this study, giving the same positive result.

Since antibodies against BSA of IgA and IgG class are frequently found in the serum of these patients, we searched for the presence of BSA in the mesangium. Two out of seven patients studied with idiotypes in the kidney also showed tiny deposits (±) of BSA in a granular pattern in the mesangial areas. The incubation of kidney biopsies of the remaining five patients with acetic acetate, pH 4, as described in Materials & Methods, did not show glomerular deposits of this antigen. The examination of renal biopsies of another five patients, belonging to the negative idiotype group, did not show deposits of BSA either in samples treated at pH 7·4 or at pH 4·0.

Relationship between renal and serum idiotypes

A comparison between idiotypes in serum and in IC and their presence in the kidney could be done in 13 patients with IgA nephropathy (Fig. 2). Blood was studied within 2 weeks of the renal biopsy. Out of eight patients showing cross-reactive idiotypes on mesangial immunoglobulins, seven (87%) pre-

sented high serum levels of idiotypes ($P < 0{\cdot}05$). Five of these patients also had increased levels of idiotype-containing IC. By contrast, only one out of five patients (20%) in whom no detectable idiotypes were observed in the mesengium showed high levels of serum idiotypes ($P < 0{\cdot}1$).

Characterization of circulating IC

In order to know the composition of circulating IC, sera from three patients with IgA nephropathy and three healthy controls were fractionated on sucrose gradient centrifugation. The existence of idiotypes and antibodies against BSA of IgG and IgA class were determined in each fraction of the gradient by ELISA, as described in Materials & Methods. As shown in Fig. 3 (upper panels) a large proportion of idiotypes was detected in fractions greater than 13 S. Analysis of the percentage of different sizes, calculated by area under rectangles, showed that fractions containing idiotype-IC with a molecular weight between 13 S and 19 S, and 19 S or greater, represented 73% (patient A), 54% (patient B) and 74% (patient C) of the total fraction in which idiotypes were detected.

Different results were obtained when the presence of anti-BSA antibodies was examined by a specific ELISA in the various fractions of the gradient (Fig. 3, lower panels). In two patients (A and C), all IgA and IgG anti-BSA antibodies were detected in 7 S fractions, or lower, therefore indicating that they were not constituents of IC. In patient B, presumably IC of small size were found, with 84% of IgA anti-BSA antibodies between 13 S and 7 S. These data are in agreement with previous results in which no correlation between serum levels of anti-BSA antibodies of different subclasses and those of serum idiotypes was found (González-Cabrero *et al.*, 1987).

DISCUSSION

In this paper we have found, by employing specific anti-idiotypic antibodies previously characterized (Gonzalez-Cabrero *et al.*, 1987), that 12 out of 42 (28%) patients with IgA nephropathy have shared idiotypes in the glomerular mesangium, with a similar pattern distribution to that of IgA. The idiotype detection was considered specific under strict criteria. The previous adsorption of rabbit antisera on an idiotype-Sepharose column completely abolished the staining. The possibility of a nonspecific binding was further excluded, because the staining also became negative when the first antiserum (rabbit anti-idiotype antibodies) was omitted or when the kidney sections were incubated instead with a normal rabbit serum. Finally, no such deposits were found in the kidney biopsies of 15 patients with membranous or mesangiocapillary glomerulonephritis incubated with the unadsorbed anti-idiotype antibodies.

Although the percentage of positive cases was relatively low, similar figures have been found in other glomerulonephritis. Thus, shared-reactive anti-DNA antibody idiotypes were observed in 19–42% of kidney biopsies of lupus patients, depending on the anti-idiotype employed to detect them (Isenberg & Collins, 1985). Theoretically, it is conceivable that in some cases an excess of anti-idiotypic antibodies, or of antigen, could cover the small amount of idiotype deposited. This last possibility was relatively excluded since BSA was only demonstrated in two out of seven patients studied with idiotypes deposited. It is also possible that, in some patients, the tiny amounts of idiotype could not be detected by immunofluorescence. In mice injected with lypopolysaccharides, T15 idiotype IC from kidney eluates represented only around 4–5% of complexes deposited (Goldman *et al.*, 1982). The possibility that antibodies were bound to structures within the renal tissue that showed homology with their idiotypes does not seem plausible, since all patients studied with other nephropathies were negative for idiotypes. Finally, it is conceivable that anti-idiotypes could react with circulating idiotype, free or complexed with the antigen, as well as with idiotypes deposited in the mesangium, in a very dynamic manner.

Although the study of kidney eluates should be of paramount importance to know the characteristics and size of deposited idiotype-containing IC, obviously it was not possible to do it in these patients. Therefore, we turned our attention upon the patients' sera. The size of IgA complexes in these patients has been studied only by some groups and in a small number of patients. Most authors agree that they are of small (7–13 S) or intermediate (13–19 S) size (Lesavre, Digeon, Bach, 1982; Valentijn *et al.*, 1983; Egido *et al.*, 1984a; Hernando *et al.*, 1986). Circulating IC containing both IgG and IgA1, with an intermediate (13–19 S) and occasionally large (> 19 S) size, have been found recently in patients with IgA nephropathy (Czerkinsky *et al.*, 1986). The profiles of idiotypic antibodies obtained by serum fractionation and ELISA show that, in three patients studied, IC containing idiotypes have chiefly a large (> 19 S) and intermediate (13–19 S) size. On the contrary, specificity to BSA could only be detected between 13–7 S and around 7 S. It is reasonable to predict that the nephritogenic potential of idiotype-bearing immune complexes might be due predominantly to a large lattice formed, perhaps together with other characteristics, like charge (Monteiro *et al.*, 1985). Idiotypes and anti-idiotypes could contribute to the formation of a critical mass of IC in the mesangium facilitating the activation of complement.

The possible pathogenic role of idiotype-anti-idiotype IC in kidney disease has been the subject of several investigations. Some mixed cryoglobulins associated with vasculitis and nephritis consist of auto-anti-idiotypes and their corresponding idiotypes (Goldman, Renverse & Lambert, 1983). Cross-reactive idiotypes have also been demonstrated in the kidneys of patients and mice with systemic lupus erythematosus (Isenberg *et al.*, 1985; Ebling & Hahn, 1985; Gavelchin & Datta, 1987). The presence of anti-idiotypic antibodies forming IC was detected in eluded kidney material from rabbits with chronic serum sickness, induced by daily injections of large amounts of BSA (Zanetti & Wilson, 1983). The close relationship observed, in the present work, between the presence of cross-reactive idiotypes on mesangial immunoglobulins and the existence of increased levels of serum idiotypes and idiotype-containing IC, suggest that the idiotype-anti-idiotype IC could play a pathogenic role in patients with IgA nephropathy. This suggestion is strengthened further by the close correlation found previously in the patients between the presence of high levels of serum idiotypes and haematuria (Gonzalez-Cabrero *et al.*, 1987).

The possible role of common food antigens such as BSA in the pathogenesis of IgA nephropathy, firstly suggested by Woodroffe *et al.* (1980), has recently been challenged (Coppo *et al.*, 1986). Although several of these antigens have been found in circulating IC and in the mesangium (Sancho *et al.*, 1983; Russel *et al.*, 1986; Yap *et al.*, 1987; Sato *et al.*, 1987), our data suggest

that idiotype-anti-idiotype IC, probably induced by continuous alimentary antigen stimulation due to the IgA immune regulation abnormalities (Egido *et al.*, 1984b), could be more pathogenic than those formed by diet antigens themselves. Thus, there was no correlation between serum levels of anti-BSA antibodies of different subclasses and those of serum idiotypes, IgA-IC or the presence of haematuria (González-Cabrero *et al.*, 1987). Furthermore, the anti-BSA antibodies were detected in different fractions of the gradient than those of idiotypes, and they barely form IC and those were of small size.

Although the antigenic specificity of idiotypic antibodies of our patients is not known, the existence of antibody molecules with quite different antigen binding specificities sharing the same idiotypes has been shown repeatedly (Abdou, 1985; Male, 1986). It is possible that the genetic predisposition to IgA nephropathy (Egido, Julian & Wyatt, 1987) may reflect, in part, the use of limited variable regions to make antibodies against bacteria and other commonly encountered antigens.

In conclusion, our findings suggest that in patients with IgA nephropathy shared idiotypes participate in the formation of circulating and renal-deposited immune complexes. The possible apposition of free anti-idiotype to idiotype already bound to glomeruli and vice versa, could contribute to increase the amount and size of mesangial immune deposits, therefore perpetuating tissue injury.

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