Clin. exp. Immunol. (1988) **73**, 395–400

Circulating heavy IgM in IgM nephropathy

S. O. DISCIULLO, J. G. ABUELO, K. MOALLI, J. C. PEZZULLO *Department of Medicine, Division of Renal Diseases, and Technology Center, Rhode Island Hospital, and Division of Biological and Medical Sciences, Brown University Providence, RI*

(Accepted for publication 4 May 1988)

SUMMARY

IgM nephropathy (IgMN) causes nephrotic syndrome and is characterized by IgM mesangial deposits. It is speculated that these deposits are derived from circulating IgM aggregates or immune complexes, either of which would have a molecular weight heavier than that of normal IgM. To test this hypothesis the sera of 11 patients with IgMN, five patients with nephrotic syndrome of other etiologies, and 13 normal controls were analysed for such heavy IgM. The serum samples were passed over a Biogel A5M molecular sieve column and the fractions were tested for IgM concentration by enzyme linked immunosorbent assay (ELISA). The column effluent from the void volume to the IgM peak was divided into four equal regions, and the average IgM concentrations in each region were compared. The IgMN group had significantly higher IgM concentrations than normal controls in the heaviest region ($0{\cdot}81 \pm 0{\cdot}84$ vs. $0{\cdot}32 \pm 0{\cdot}17$ μg/ml; $P = 0{\cdot}01$) and in the lightest region ($95{\cdot}8 \pm 59{\cdot}5$ vs. $46{\cdot}3 \pm 41{\cdot}2$ μg/ml; $P = 0{\cdot}02$). Although the IgMN group appeared to have about double the IgM levels of the nephrotic control group in all four regions, this was only significant in the lightest (19S) region. In serum samples from two IgMN patient methods known to break antigen antibody bonds eliminated the heavy IgM; in one case we used gel filtration in potassium thiocyanate and in another ultracentrifugation at pH 2·8. In addition, the heavy IgM in this second patient exhibited complement fixation activity in a sandwich ELISA for IgM-C3 complexes. We conclude that IgMN patients have circulating heavy IgM, which by preliminary studies probably consists of complement fixing IgM immune complexes.

Keywords immune complexes IgM nephropathy nephrotic syndrome mesangial proliferative glomerulonephritis, IgM

INTRODUCTION

IgM nephropathy (IgMN) has recently been described as a cause of nephrotic syndrome (Bhasin *et al.*, 1978; Cohen, Border & Glassock, 1978). The disease is characterized immunohistologically by diffuse global mesangial deposition of IgM, which is the predominant or only immunoglobulin present, and morphologically by a mild to moderate diffuse increase in mesangial cellularity and matrix. It may develop secondarily in patients with lipoid nephrosis (Tejani, 1985; Hirszel *et al.*, 1984) and can progress to focal glomerulosclerosis (Cohen *et al.*, 1981; Allen *et al.*, 1982; Gurumurthy, Tejani & Nicastri, 1983; Hirszel *et al.*, 1984; Ji-Yun *et al.*, 1984; Pardo *et al.*, 1984. In the absence of any real information about the cause or pathogenesis of IgMN, several questions have remained unanswered. Is it truly a separate disease? What is the relationship of IgM nephropathy to lipoid nephrosis and focal glomerulosclerosis? Should the diagnosis IgMN be applied to patients with mesangial IgM deposition in the absence of mesangial hypercellularity (Cavallo & Johnson, 1981; Cohen *et al.*, 1981; Allen *et al.*, 1982, Vilches *et al.*, 1982; Gurumurthy, Tejani & Nicastri, 1983; Helin *et al.*, 1982; Hsu *et al.*, 1984; Pardo *et al.*, 1984; Ji-Yun *et al.*, 1984; Looi *et al.*, 1985) or in the absence of nephrotic syndrome, but in the presence of atypical clinical features such as isolated or gross hematuria (Cohen *et al.*, 1981; Helin *et al.*, 1982; Hirszel *et al.*, 1984)?

Perhaps the most fundamental question concerns the pathogenic role and nature of IgM in this condition. There is some evidence that the presence of IgM deposits can be correlated with steroid nonresponsive disease (Cohen *et al.*, 1981; Allen *et al.*, 1982; Tejani & Nicastri, 1983) and that the prognosis of nephrotic patients with IgM deposits may not be as favorable as the prognosis in those without deposits (Cohen *et al.*, 1981; Gurumurthy, Tejani & Nicastri, 1983). However, others maintain that the presence of mesangial IgM deposits in these patients has no effect on the response to treatment or the outcome of the disease process (Vilches *et al.*, 1982; Pardo *et al.*, 1984; Ji-Yen *et al.*, 1984).

Correspondence: J. Gary Abuelo, M.D. Rhode Island Hospital 593 Eddy Street, Providence, RI 02902 USA

With regard to the nature of the mesangial IgM, it may represent the deposition of circulating antigen-IgM antibody complexes. Supporting this idea are the following: 1 the presence of IgM containing complexes in the sera of some patients (Helin *et al.*, 1982, Lin & Chu, 1986); 2 the *in vitro* fixation of heterologous complement to the mesangial areas, and the susceptibility of the IgM deposits to acid elution (Cavallo & Johnson, 1981); 3 the deposition of circulating IgM containing immune complexes in the kidneys of experimental animals (Brown & Harkiss, 1981; Border & Cohen, 1983); and 4 in many cases of IgMN the presence of electron-dense granular deposits that are typical of immune complex deposition (Bhasin *et al.*, 1978; Cohen, Border & Glassock, 1978; Cavallo & Johnson, 1981, Mampaso *et al.*, 1981; Allen *et al.*, 1982; Helin *et al.*, 1982; Hirszel *et al.*, 1984; Hsu *et al.*, 1984; Pardo *et al.*, 1984; Looi *et al.*, 1985). Similarly, the mesangial IgM could be deposition of circulating aggregates of IgM as has been observed after the injection of artifically aggregated human IgM into rats (Kijlstra, *et al.*, 1978). Alternatively, glomerular IgM may be a result of a defect in the normal clearing of plasma proteins, including IgM, from the mesangium (Cavallo & Johnson, 1981; Mampaso *et al.*, 1981). One possible defect could be the widening and increased volume of mesangial channels which would allow for the pooling and deposition of IgM (Grond & Elema, 1981; Olivetti *et al.*, 1981). If patients with IgMN have such a defect, mesangial IgM deposition would not depend on abnormal quality and quantity of serum IgM.

The purpose of this study, therefore, was to detect heavy IgM (either immune complexes or aggregates) in the sera of patients with IgMN and to determine if the presence or absence of circulating heavy IgM could be correlated with severity of clinical disease. In addition, we demonstrated that the 'heavy' IgM in the sera of two patients could be dissociated by conditions known to break antigen antibody bonds (Benveniste & Bruneau, 1979).

MATERIALS AND METHODS

Subjects

Thirteen sera were obtained from 11 patients who initially had nephrotic syndrome due to IgMN. Five of these sera came from four patients who still had nephrotic syndrome, while eight sera came from seven patients in partial or complete remission. Eight patients were males; three were females. The average age was 13·3 years (range 2 to 26 years). The diagnosis was based on renal biopsies that showed slight to marked diffuse mesangial hypercellularity by light microscopy and diffuse mesangial deposition of IgM as the sole or predominant immunoglobulin by immunofluorescent microscopy. Electron microscopy showed fusion of the foot processes over variable segments of the capillary walls and the mesangial cell increase. Electron dense deposits were seen in the mesangium in six of the eleven patients.

Sera from 13 normal individuals aged 6 to 31 years served as normal controls and sera from five nephrotic patients with diverse renal diseases were disease controls. These five individuals included one case each of diabetic nephropathy, lipoid nephrosis, membranoproliferative glomerulonephritis, diffuse proliferative form of lupus nephritis and one elderly man without a tissue diagnosis.

Total Serum IgM Concentrations

Serum IgM concentrations were performed on all sera by nephelometry on a Auto-ICS nephelometer (Beckman Instruments Incorporated, Brea, CA).

Chromatography

One millilitre fractions of each serum were subjected to gel filtration chromatography on a Biogel A5M (Biorad Laboratories, Rockville Centre, New York) 100 × 2·5 cm column equilibrated in phosphate-buffered saline (PBS: 0·15 M NaCl, 0·01 M potassium phosphate buffer, pH 7·4). In addition, as a preliminary experiment serum from one patient with heavy IgM was divided into two 0·6 ml samples, which were dialysed overnight, one against PBS and the other against 0·5 M potassium thiocyanate (KSCN). The samples were filtered through a 0·45 μm membrane filter (HA type, Millipore Corporation, Bedford, Massachusetts) and passed over Sepharose CL-4B (Pharmacia Fine Chemicals Inc., Piscataway, NJ) 70 × 2·5 cm columns equilibrated with the respective solutions. The void volume was determined by the addition of 5 mg of blue dextran to each serum sample. Fractions of 5 ml were collected, examined spectrophotometrically at 280 nm, and assayed for IgM using an enzyme immunoassay.

Ultracentrifugation

Serum samples of 0·1 ml were layered on a 10–40% linear 10 ml sucrose gradient using either 0·12 M glycine HCl, pH 2·8 (Beneveniste & Bruneau, 1979), or 0·02 M potassium phosphate, pH 7·3, as diluting buffers. They were centrifuged in an LC-50 ultracentrifuge using an SW 27 rotor (Beckman Instruments, Inc., Palo Alto, California) for 28 h at 75,000 *g*. Fractions of 0·33 ml were collected and assayed for the presence of IgM, and IgM-C3 complexes using enzyme immunoassays.

Enzyme immunoassays

Gel fractions from the void volume to the IgM peak and ultracentrifuge fractions were assayed for the presence of IgM by an IgM enzyme immunoassay. Polystyrene tubes (Sarstedt, Princeton, New Jersey) were coated with 1 μg of affinity purified goat anti-human IgM (Antibodies Incorporated, Davis, California) in 1 ml of 0·01M sodium carbonate-bicarbonate buffer, pH 9·6. The tubes were allowed to stand for 3 h at room temperature followed by overnight incubation at 4°C. The solution was discarded and the tubes were washed three times with phosphate-buffered saline-Tween (PBS-Tween: 0·15 M NaCl, 0·1M potassium phosphate buffer, pH 7·5, 0·05% Tween 20). One millilitre of an appropriate dilution of each serum fraction was added to the antibody coated tubes and allowed to stand for 2 h at room temperature. The tubes were washed three times with PBS-Tween. This was followed by the addition of 1 ml of horseradish peroxidase conjugated goat anti-human IgM (Litton Bionetics Inc., Kensington, Maryland) diluted 1 : 700 in PBS-Tween. After incubating 2 h at room temperature, the tubes were washed three times with PBS-Tween and developed with 1 ml of substrate solution (26·2 mg O-phenylene-diamine, 0·57 ml 3% hydrogen peroxide, 100 ml 0·1 M citrate-phosphate buffer, pH 5·5). The enzymatic reaction was stopped after 30 min at room temperature by the addition of 0·1 ml 2·5 M sulfuric acid and read on a spectrophotometer at 492 nm. Standard curves were prepared by diluting immunoglobulin standards

(Kent Laboratories, Redmond, Washington) in PBS-Tween to IgM concentrations from 1000 ng/ml to 1·9 ng/ml.

A C3-IgM sandwich enzyme immunoassay was used to detect complement fixation by heavy IgM in sera fractionated by ultracentrifugation. Microtitre plates (Immuno Plate II, Nunc, Denmark) were coated with 0·1 mg of monospecific goat IgG anti-human C3 (Cooper Biomedical Inc., Halvern, Pennsylvania) in 0·1 ml 0·01 M sodium carbonate-bicarbonate buffer, pH 9·6, as described for polystyrene tubes above. The plates were washed three times with PBS-Tween, followed by the addition of 0·1 ml of an appropriate dilution of each fraction to the wells. After a 2 h incubation at room temperature, the wells were washed three times with PBS-Tween and 0·1 ml of horseradish peroxidase labelled goat anti-human IgM diluted 1 : 700 in PBS-Tween was added. Two hours later, the anti-IgM was aspirated, the wells were washed with PBS-Tween and the colour was developed by the addition of 0·1 ml O-phenylenediamine solution (see above). The reaction was stopped after 20 min by the addition of 10 μl 2·5 M sulfuric acid. The colour intensity was read on a Minireader II (Dynatech Laboratories, Inc., Alexandria, Virgnia) at a wavelength of 490 nm.

Heavy IgM determinations

The IgM concentrations in each test tube from the void volume to the IgM peak were recorded in a microcomputer, which divided the column effluent in this area mathematically into four regions of equal volume: region 1, which began at the void volume, to region 4, which ended at the IgM peak. An average IgM concentration was determined for each of the four regions in each subject. The amount of heavy IgM in an individual's serum was assumed to be reflected in the IgM concentrations in the three heaviest regions: 1, 2 and 3.

Statistical analysis

The IgM concentrations of the IgMN patients in each region were compared to concentrations of the controls by Student's *t*-test. Results are expressed as mean $\pm$ standard deviations.

RESULTS

Total serum IgM concentrations were measured in IgMN patients and controls. IgMN patients had 191 $\pm$ 116 mg/dl. This was significantly higher than the levels in nephrotic controls (116 $\pm$ 158 mg/dl; $P < 0{\cdot}01$), but not statistically different from levels in normal controls (159 $\pm$ 191 mg/dl).

Presence of heavy IgM

A typical graph of IgM concentrations in fractions of a normal serum chromatographed on a Biogel A5M gel filtration column is shown in Fig. 1. The fractions from the void volume to the 19S peak were divided into four equal regions. When the IgMN patients' mean concentrations of IgM in each region were compared to those of the normal controls, it was observed that the patients had significantly higher levels of IgM in regions 1 and 4 (19S), while no differences between the two groups were found in regions 2 and 3 (Table 1). These results are evidence that IgMN patients not only have higher levels of 19S IgM than normal controls, but also possess more IgM in the heaviest region, region 1.

The concentration of IgM in region 1 was greater in four IgMN patients with nephrotic syndrome (1·29 $\pm$ 1·16 μg/ml) than in seven IgMN patients in partial or complete remission (0·51 $\pm$ 0·37 μg/ml), but this difference was of marginal significance ($P = 0{\cdot}1$). The four IgMN patients on prednisone at the time of the sample had no significant difference in IgM levels in

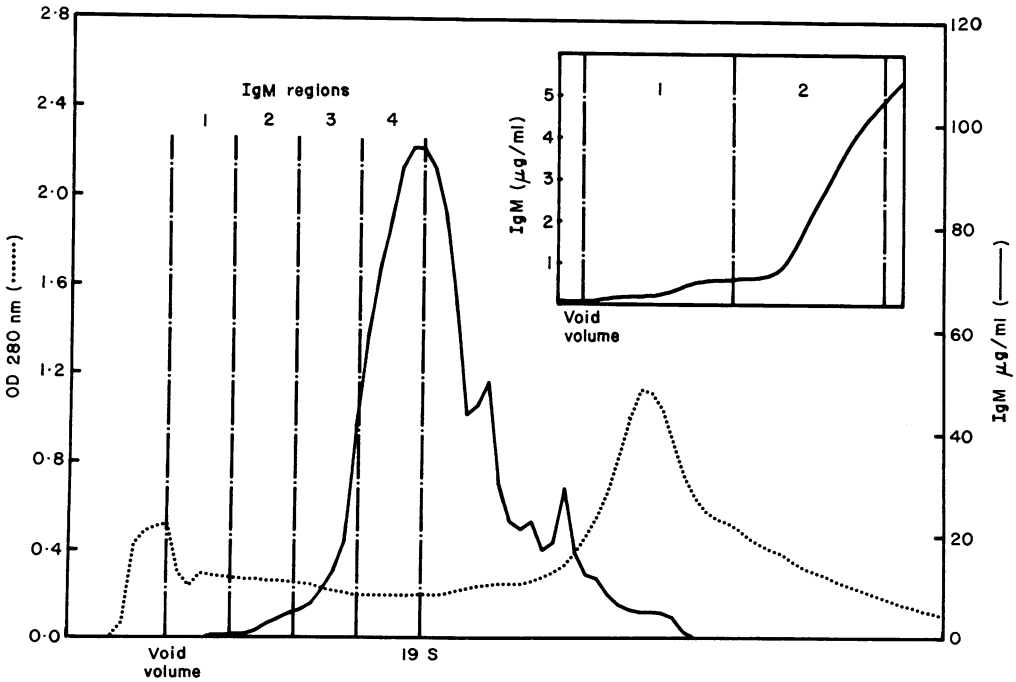

Fig. 1. Chromatography of normal serum on a Biogel A5M gel filtration column. The solid line shows IgM concentration. The four IgM regions are indicated.

Table 1. IgM concentration in fractions of sera chromatographed on a Biogel A5M gel filtration column. Results are expressed as average concentrations within the four regions (see text) in μg/ml.

	IgM Region			
	1	2	3	4(19S)
Normals (n=13)	0·32±0·17	0·84±0·50	4·73±2·30	46·3±41·2
IgM nephropathy (n=13)	0·81±0·84	1·47±1·20	8·04±5·90	95·8±59·5
P value	0·011	NS	NS	0·019

NS not significant.

regions 1 and 4 when compared to the seven IgMN patients not taking prednisone.

Patients with nephrotic syndrome of other etiologies were also assayed for levels of IgM in the four regions. These levels were about half of those in the IgMN group in all regions (data not shown). However, only the difference in the 19S region, region 4, was found to be statistically significant (38·1±12·2 μg/ml, $P<0·025$), possibly due to the small number (five) of nephrotic patients.

Nature of heavy IgM

To determine if the heavy IgM existed as either immunoglobulin aggregates or immune complexes, serum with heavy IgM from a patient with IgMN was dialysed against KSCN and chromatographed over a Sepharose CL-4B column equilibrated in the same solution. As can be seen in Fig. 2, exposure to KSCN eliminated the heavy IgM seen when dialysis and chromatography were carried out with PBS. Similarly, serum from another IgMN patient showed dissociation on ultracentrifugation at pH 2·8 of the heavy IgM seen with ultracentrifugation at pH 7·3 (Fig. 3a). Complement fixation by the heavy IgM was demonstrated in the heavy fractions by a positive sandwich assay for IgM-C3 complexes; these complexes were no longer detected after ultracentrifugation at pH 2·8 (Fig. 3b).

DISCUSSION

The significance of mesangial IgM in patients with nephrotic syndrome and minimal glomerular abnormalities has long been a matter of controversy. The histologic and clinical criteria for diagnosing IgMN, the effect of mesangial IgM on prognosis and response to therapy, and the relation of IgMN to lipoid nephrosis are questions that cannot be fully resolved until the genesis of the IgM deposits is understood.

It has been suggested that IgM deposition may be a result of circulating IgM containing immune complexes or IgM aggregates. Our study presents evidence that patients with IgMN may have increased amounts of circulating heavy IgM. Dissociation of this heavy IgM by 0·12 M glycine HCl, pH 2·8, in one case and by 0·5 M KSCN in another case is compatible with the hypothesis that the IgM is in the form of circulating immune complexes. This is further supported by our observation of acid dissociable C3-IgM complexes in one of these cases. On the

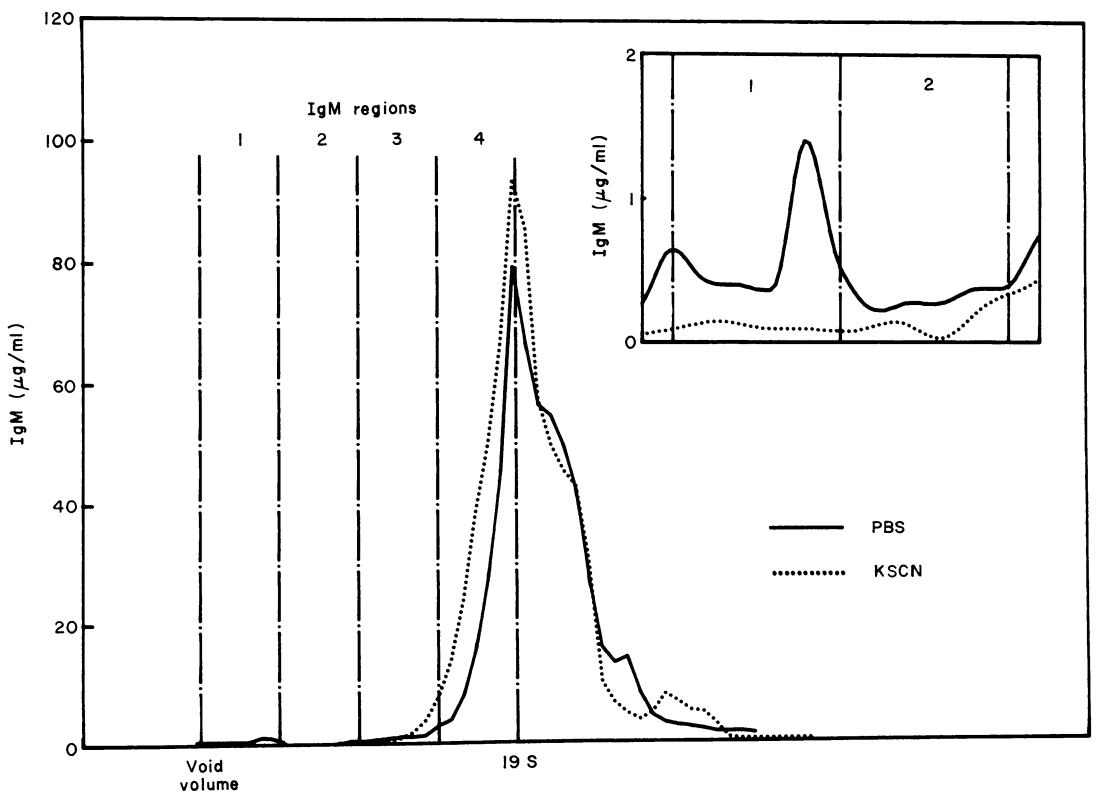

Fig. 2. Effect of KSCN on heavy IgM chromatography of serum from a patient with IgMN shows reduction of IgM levels in regions 1 and 2 after exposure to KSCN.

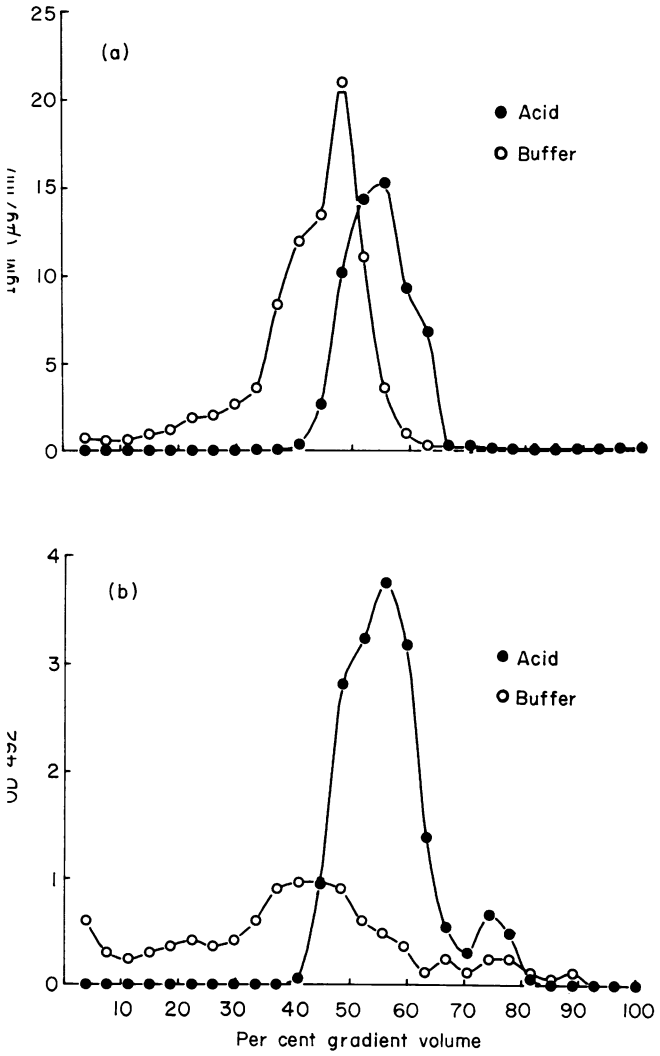

Fig. 3. Fractionation by ultracentrifugation in acid and PBS of serum from a patient with IgMN. a. Marked reduction of heavy IgM concentration by acid. b. Marked reduction of IgM-C3 complexes by acid.

other hand, non-specifically aggregated IgM could also dissociate under these conditions, although they often do not (Benveniste & Bruneau, 1979). These dissociation studies in two cases cannot automatically be generalized to all IgMN patients. However, taken together with the results of Helin *et al.* (1982), who reported the presence of IgM immune complexes in the serum of five patients with IgMN using a C1q solid phase ELISA test, and those of Lin and Chu (1986), who found IgM immune complexes in eight out of 11 IgMN patients at the onset of disease, they provide evidence that the heavy IgM is in the form of immune complexes.

High levels of circulating IgM immune complexes may reflect an increased production of such complexes. This might be seen if IgMN patients tended to respond to antigenic challenges with augmented and predominantly IgM responses. This explanation is consistent with the following observations: 1 few IgMN patients have circulating IgG complexes (Helin *et al.*, 1982; Lin & Chu, 1986); 2 high serum 19S IgM levels were found in our IgMN patients; and 3 elevations of both total IgM concentration (Mapaso *et al.*, 1981; Helin *et al.*, 1982; Hsu *et al.*, 1984; Lin & Chu, 1986) and IgM-bearing peripheral lymphocytes (Lin & Chu, 1986) have been previously reported in IgMN.

The global increase of IgM production may be explained by an abnormality in B cell differentiation which, in turn, may be due to a defect in regulatory T cell function. It has been demonstrated that T cell derived lymphokines affect many aspects of B cell growth and differentiation. Recently, two lymphokines have been described which mediate the differentiation of B cells into IgM or IgG-secreting cells (Kishimoto, 1985). An excess of factors that induce IgM secretion or a defect in lymphokine activity responsible for switching B cells to IgG production would explain increased levels of IgM in IgMN patients.

Alternatively, reduced clearance of circulating antigen IgM antibody complexes could also explain the high serum levels observed. These complexes have been shown to be mainly taken up by the liver (Brown & Harkiss, 1981). A defect in this clearance mechanism in patients with IgMN could lead to accumulation in the circulation of the small amounts of IgM immune complexes that may be formed normally through low grade exposure to dietary, microbial or other antigens.

One must also consider the possibility that patients with IgMN not only have increased IgM complexes, but also have abnormalities of the mesangium which facilitate the accumulation of IgM immune complexes. For example, there may be increased entry of macromolecules into the mesangium due to glomerular injury (Mauer *et al.*, 1974) or to angiotensin II or other vasoactive peptides (Raij & Keane, 1985). Alternatively, there may be pooling of macromolecules due to widened mesangial channels (Olivetti *et al.*, 1981; Grond & Elema, 1981) or impaired clearance of macromolecules due to immune mediated glomerular disease (Keane & Raij, 1980).

The significance of IgM immune complexes in IgMN depends to some degree on the specificity of this finding. For example, in the case of IgG immune complexes, which have been found in over 100 different diseases, a positive assay result is difficult to interpret and the clinical usefulness of the test is questionable (Agnello, 1981; Abuelo *et al.*, 1982). Circulating IgM complexes have been detected in systemic lupus erythematosis (Hautanen & Linder, 1979), viral hepatitis A and B (Margolis *et al.*, 1987), and in a minority of cases of IgA nephropathy, Henoch–Schoenlein nephritis, membranous glomerulonephritis and lupus nephritis (Coppo, *et al.*, 1982; Doi *et al.*, 1982). On the other hand, no IgM immune complexes were detected in 20 patients with lipoid nephrosis (Lin, 1985); and in an unpublished study we found that heavy IgM levels were no higher in 57 hospitalized patients with a variety of diseases than in 31 normal controls. Thus, IgM immune complexes are by no means specific for IgMN. However, their absence in lipoid nephrosis which has no IgM mesangial deposits, but otherwise resembles IgMN, is evidence for their pathogenic role in IgM mesangial deposition.

In conclusion, our results taken together with previous studies suggest that IgMN is characterized by circulating complement fixing IgM immune complexes in concentrations significantly higher than those of normal or nephrotic controls. This finding is compatible with but not proof of the hypothesis that IgMN is a separate disease entity. It also suggests that the origin of mesangial IgM in this condition is by deposition from the increased amount of IgM immune complexes circulating in the plasma.

ACKNOWLEDGMENTS

We thank Charlene McGloin for her secretarial assistance and Dr Andrew Brem for providing many of the serum samples.

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