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Immunohistochemical study of the membrane attack complex of complement in IgA nephropathy

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Summary. The localization of the membrane attack complex of complement (MAC) was examined in the normal human kidneys and in biopsy specimens from patients with primary IgA nephropathy by immunofluorescent and immunoelectron microscopies. Immunofluorescent staining for MAC was significantly more intense than in the normal kidneys, and was observed in the mesangium and occasionally along the glomerular capillary walls of 22 of 30 patients with IgA nephropathy. By dual-staining, the MAC deposits were generally concordant with the deposits of IgA, C3, C5 and C9, or of IgG, when present. $C1_q$ or C4 was infrequently observed in the glomeruli. Immunoelectron microscopy revealed various staining patterns of glomerular MAC deposition; homogeneous fine-granular staining beneath the glomerular basement membrane (GBM) in the paramesangial zone, patchy staining within the mesangial electron dense deposits (EDD), and ring-shaped or ribbon-like staining, associated with the striated membrane structures (SMS), in the matrix of the mesangium, GBM and tubular basement membrane (TBM). This study suggests that the terminal complement system is activated, mainly by an alternative complement pathway mechanism, in the mesangium of IgA nephropathy, and is associated with the paramesangial lesion and EDD. MAC deposition in glomerular SMS may also result from in situ activation rather than trapping from the circulation. There was little correlation between glomerular MAC deposition and proteinuria or renal histology of patients with IgA nephropathy.

Key words: IgA nephropathy – Membrane attack complex – Complement – Striated membrane structures

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Introduction

Primary IgA nephropathy (Berger and Hinglais 1968) although the frequency varies greatly among different population (Egido et al. 1983) is detected in approximately 40% of the kidney biopsy specimens in Japan (Nakamoto et al. 1978). There is increasing evidence that this disease is progressive, and is now considered to be an important cause of renal failure. Several studies have suggested that IgA nephropathy is mediated by deposition of IgA polymers from the circulation. The level of immune complex containing IgA was elevated in the sera of patients with IgA nephropathy (Lopez-Trascasa et al. 1980; Woodroffe et al. 1980). IgA deposition with IgG and C3 is found in the renal mesangium (Dysart et al. 1980; Egido et al. 1983), subcutaneus blood vessels (Baart de la Faille-Kuyper et al. 1976), and transplanted kidneys (Berger et al. 1975). The mesangial IgA deposits are of polymeric size, and can bind the free secretory component (Bene et al. 1982; Tomino et al. 1982; Valentijn et al. 1984). However, the importance of immune deposits and the role of the complement system in renal injury remains unknown.

Recently, the presence of the membrane attack complex of complement (C5b-9) (MAC) has been demonstrated in the kidney tissue of normal subjects, and patients or animals with various forms of renal diseases (Biesecker et al. 1981; Falk et al. 1983; Koffler et al. 1983; Adler et al. 1984; Perkinson et al. 1985; Cosyns et al. 1986; Hinglais et al. 1986). MAC is formed after cleavage of C5 by classical or alternative complement pathway mechanisms, and has a characteristic cylindrical appearance with an internal diameter of 10 nm, an external diameter of 21 nm, and a length of 16 nm (Podack and Tschopp 1984). In both normal and diseased kidney tissues, MAC is frequently localized

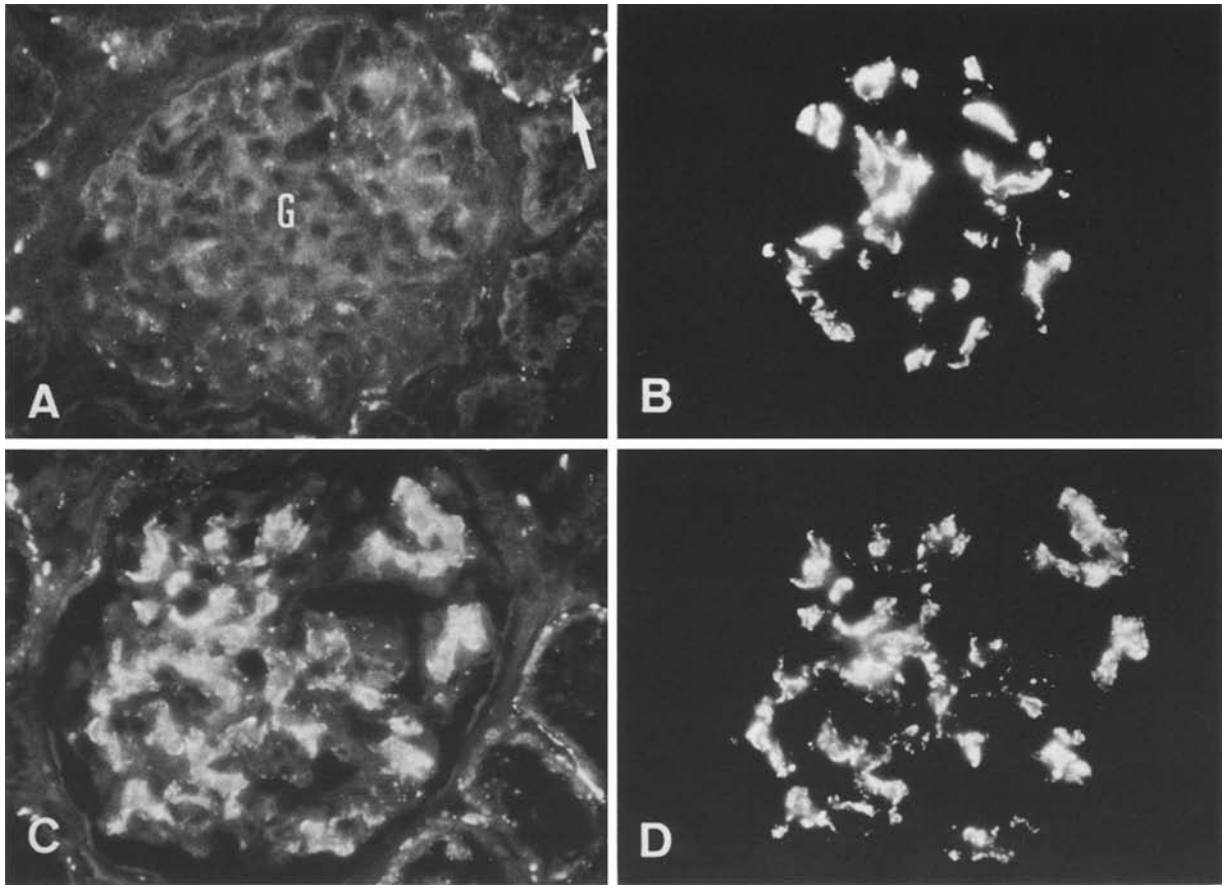

Fig. 1. Immunofluorescent micrographs of kidney sections stained for MAC (**A** and **C**), IgA (**B**) and C3 (**D**). (**A**) Normal human kidney. MAC is stained granularly along the TBM (*arrow*), and weakly in a granular pattern in the glomerular mesangium. (**B–D**) IgA nephropathy. IgA is localized predominantly in the mesangium (**B**). By the dual-staining with Poly C9-MA and anti-C3 antibody, MAC (**C**) and C3 (**D**) show co-distribution in the mesangium, but they are dissociated along the TBM. *G*: glomerulus. Original magnification: **A–D**, ×400

in the glomerular basement membrane (GBM) and mesangial matrix, correlated with phase dense materials by phase contrast microscopy (Falk et al. 1983), or with round extracellular particles and striated membrane structures (SMS) by immunoelectron microscopy (Hinglais et al. 1986; Falk et al. 1987). The association between MAC and immune deposits located in the glomerular subendothelial or subepithelial space, or in the mesangium has been demonstrated in several types of human glomerulonephritis (Biesecker et al. 1981; Falk et al. 1983; Cosyns et al. 1986; Hinglais et al. 1986; Falk et al. 1987). Studies of experimental membranous nephropathy induced by immunization of normal and C6-deficient animals with cationic bovine serum albumin suggested that MAC is responsible for tissue damage and proteinuria (Groggel et al. 1983). In addition to the well-known haemolytic action, MAC has been reported to have non-lytic cytopathic effects on nucleated cells by Yamamoto and Wilson (1986) who studied the role of MAC in anti-Thy-1 antibody mediated mesangial cell injury.

To clarify the role of the terminal complement pathway in the pathogenesis of IgA nephropathy, we examined the localization of MAC in kidney tissue sections by immunofluorescent and immunoelectron microscopies.

Materials and methods

Tissue samples consisted of routine renal biopsy specimens taken from 30 patients (21 males and 9 females, mean 11 years, between 4 and 17 years old) with primary IgA nephropathy. The diagnosis was made based on clinical, immunofluorescence, and light microscopic findings. All patients had macro- or microscopic haematuria and proteinuria. One patient had nephrotic syndrome. Twenty eight patients had normal renal function, and two had moderate renal failure. None of them were hypertensive and none had clinical or biochemical evidence of Henoch-Schonlein purpura nephritis, systemic lupus erythematosus, or liver disease.

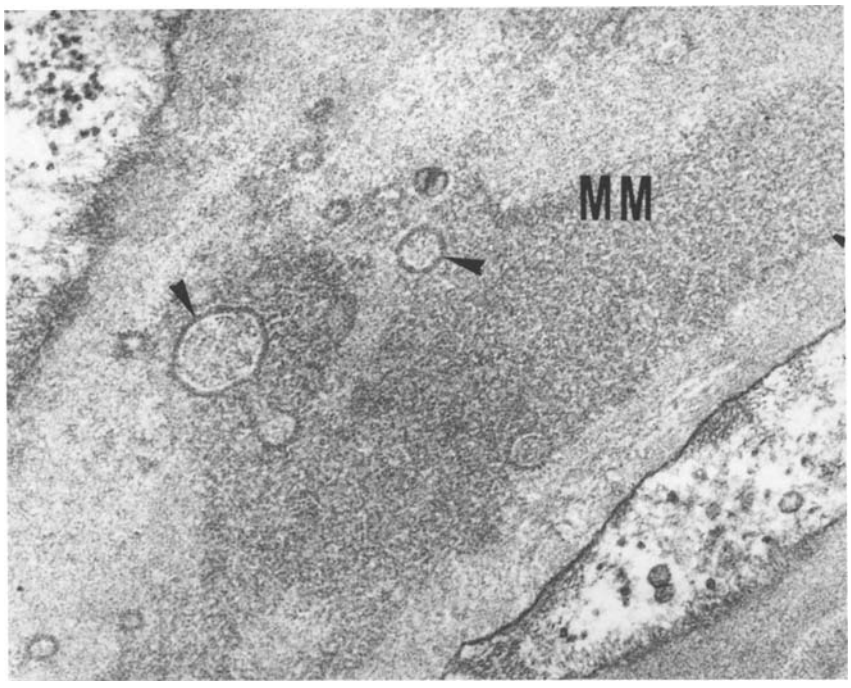

Fig. 2. Conventional electron micrograph illustrating SMS (arrow heads) in the mesangial matrix (MM). Original magnification: ×25000

The pathological changes in light microscopy included mild (one patient) or moderate (29 patients) diffuse mesangial proliferation, with (6 patients) or without (24 patients) segmental accentuation. Glomerular crescents (10–28%) were found in the tissue of six patients. The histologically normal portion of the kidney tissue obtained from four patients (43, 47, 50 and 66 years old) with renal trauma, renal calculus or renal tumours were used as normal controls.

A monoclonal antibody (Poly C9-MA) (mouse IgG_1) to a neoantigen of the C9 portion of MAC was kindly provided by Dr. A.F. Michael, University of Minnesota (Falk et al. 1983). The following polyclonal antibodies were purchased from Cappel (Malvern, PA, USA); goat antisera to human IgG, IgA, C3, C4, C5, and C9. Fluorescein-isothiocyanate (FITC)-labeled $F(ab')_2$ fragments of goat antisera to human IgG, IgA, IgM and C3, and FITC-conjugated IgG fraction of goat antisera to human C4 were also purchased from Cappel. The anti-C9 antiserum was absorbed with C9 deficient human serum (provided by Dr. S. Inai, Osaka Medical School, Osaka, Japan). The specificity of the antisera was checked by double gel immunodiffusion (Ouchterlony) and immunoelectrophoresis. The IgG fraction was prepared from the unlabeled polyclonal antisera to IgG, IgA, C3, C4, C5 and C9 by protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column chromatography, or by ammonium sulfate precipitation followed by DEAE cellulose column chromatography. After treatment with pepsin, the Fab fragment of IgG was obtained by Sephadex G-200 (Pharmacia) gel filtration, and conjugated with horse-radish peroxidase (HRP) by the periodate oxidation method (Nakane and Kawaoi 1974). The FITC-, tetramethy rhodamine (TRIC)-, or HRP-labeled $F(ab')_2$ fragment of goat antisera to mouse IgG (Cappel), and FITC-conjugated IgG fraction of rabbit antisera to goat IgG (Cappel) were preabsorbed with normal human plasma, and used as the secondary antibody in immunofluorescent or immunoperoxidase staining.

Direct or indirect immunofluorescence microscopy of 4 μm-thick cryostat sections was performed as previously described (Yoshioka et al. 1985; Takemura et al. 1987). MAC was stained by an indirect method using Poly C9-MA as the first antibody and FITC-labeled $F(ab')_2$ goat anti-mouse IgG as the secondary antibody. C5 and C9 were stained with the IgG fraction of goat anti-C5 or anti-C9 antisera and FITC-labeled IgG fraction of rabbit antiserum to goat IgG. IgA, IgG, C3 and C4 were stained by a direct method. Dual-staining was carried out as described elsewhere (Takemura et al. 1987; Yoshioka et al. 1987a); tissue was incubated with FITC-labeled goat antisera to human IgA, IgG, C3 and C4, or unlabelled antibody to C5 and C9 with the appropriate secondary antibody conjugated FITC, and then reacted with Poly C9-MA and TRIC-labeled goat anti-mouse IgG antibody. Intensity of the antibody staining was scored in a semiquantitative manner (negative, trace, 1+ or 2+).

Direct (IgG, IgA, C3, C4, C5 and C9) and indirect (MAC) peroxidase staining was performed as described previously (Takemura et al. 1987). In brief, the tissue was fixed in paraform aldehyde-lysine-periodate fixative of McLean and Nakane (1974), and progressively treated with sucrose in phosphate buffered saline (PBS). After being cut into 4 μm-thick sections, the specimen was treated with periodate and sodium borohydroxide to inhibit endogenous peroxidase. In the direct staining, the HRP-labeled Fab fragment of antisera was overlaid on the tissue section at 4° C overnight. In the indirect staining, the section was initially reacted with poly C9-MA at 4° C overnight, and then incubated with HRP-labeled goat anti-mouse IgG at room temperature for 45 min. After staining, the tissue was fixed with 1% glutaraldehyde/PBS, incubated with the substrate solution, diaminobenzidine, and then fixed with osmic acid. The tissue was cut into ultrathin sections, and observed under an electron microscope (Hitachi Model HU 12A) without counter-staining.

After fixation in glutaraldehyde, the tissue was washed with cold buffer and postfixed in osmium tetroxide. Epon-embeded ultrathin sections were stained with uranyl acetate and lead citrate, and then examined by electron microscopy.

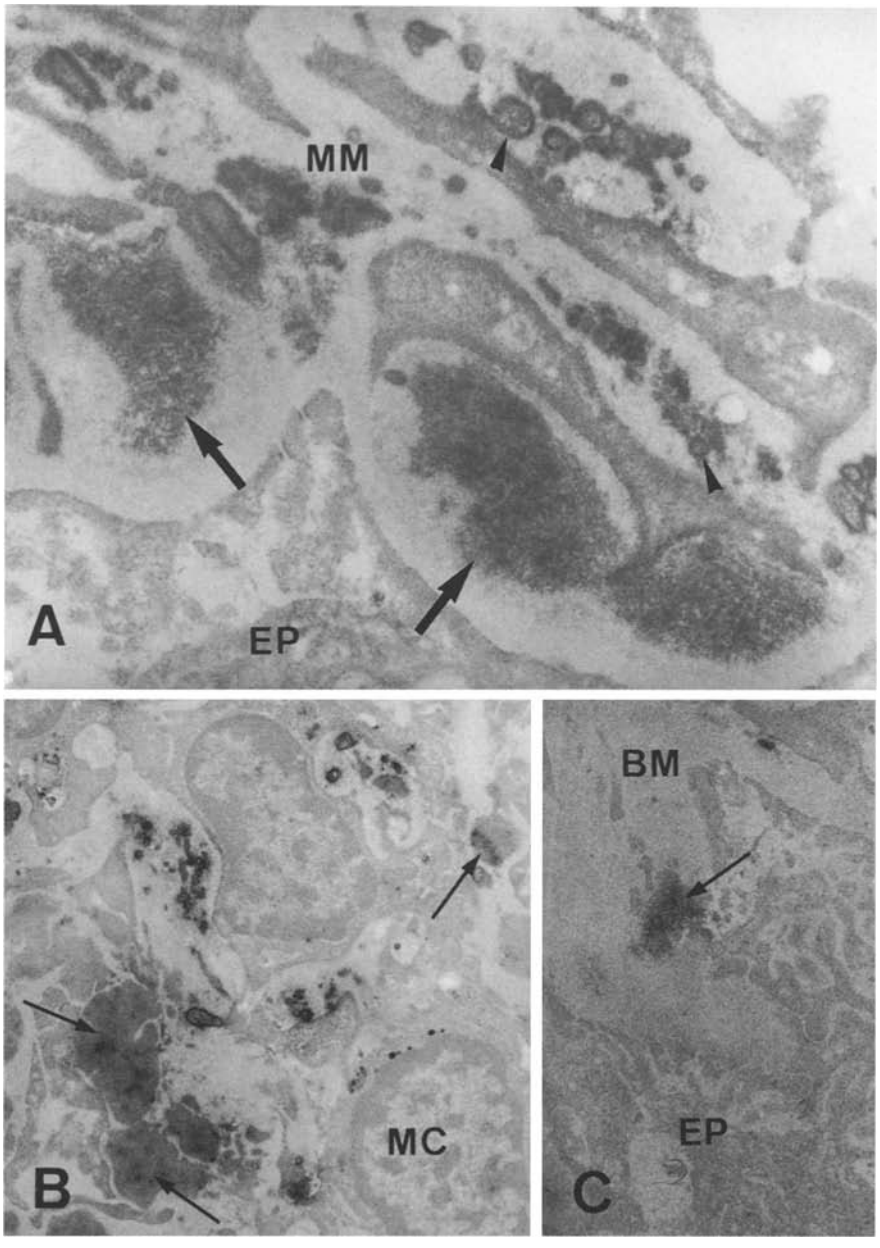

Fig. 3. Immunoelectron micrographs of glomeruli from patients with IgA nephropathy, stained for localization of MAC. (**A**) MAC staining in a homogeneous, fine-granular pattern (*arrows*) beneath the GBM in the paramesangial zone, and MAC staining associated with SMS in the mesangial matrix (arrow heads). (**B**) Patchy staining of MAC (*arrows*) within the mesangial EDD. (**C**) Subepithelial MAC staining (*arrow*). Original magnification: **A** ×10000, **B** ×3500, and **C** ×5000. *MM*: mesangial matrix. *EP*: glomerular epithelial cell. *MC*: mesangial cell. *BM*: glomerular basement membrane

Results

In the normal human kidney sections, Poly C9-MA was fixed in a granular pattern, to the segments of tubular basement membrane (TBM) and the wall of blood vessels, and weakly to the glomerular mesangium (Fig. 1 A) in immunofluorescent preparations.

Glomerular MAC deposition (as interpreted by reactivity with Poly C9-MA) with significantly higher intensity and wider distribution than in normals was found in 22 of the 30 patients with IgA nephropathy (Fig. 1 C). MAC was predominantly localized in the mesangial area, and occasionally deposited along the glomerular capillary walls. MAC was also detected in the sclerosed area of the glomeruli, but not in the glomerular crescents. Coarse-granular staining with Poly C9-MA was seen along the TBM of 28 of the 30 patients. Staining for IgG, C3 and C4 was positive in the glomeruli of 23, 19 and 2 of the 30 patients, respectively. Dual-staining of MAC (TRIC) and IgA, IgG, C3, C5 or C9 (FITC) was performed in the tissue section of five patients (Fig. 1 C and D). MAC was concordant with IgA, C3, C5 and C9, or IgG when present, in four patients, but partly discordant with

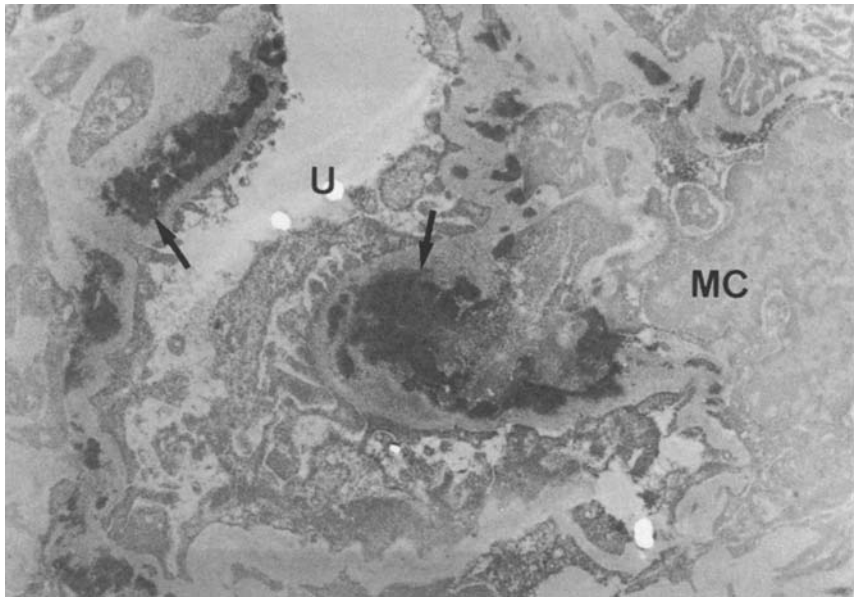

Fig. 4. Immunoelectron micrograph of a portion of a glomerulus from a patient with IgA nephropathy, stained for IgA. Homogeneous reaction products are prominent along the GBM in the paramesangial zone (*arrows*). Original magnification: ×3000. *U*: urinary space

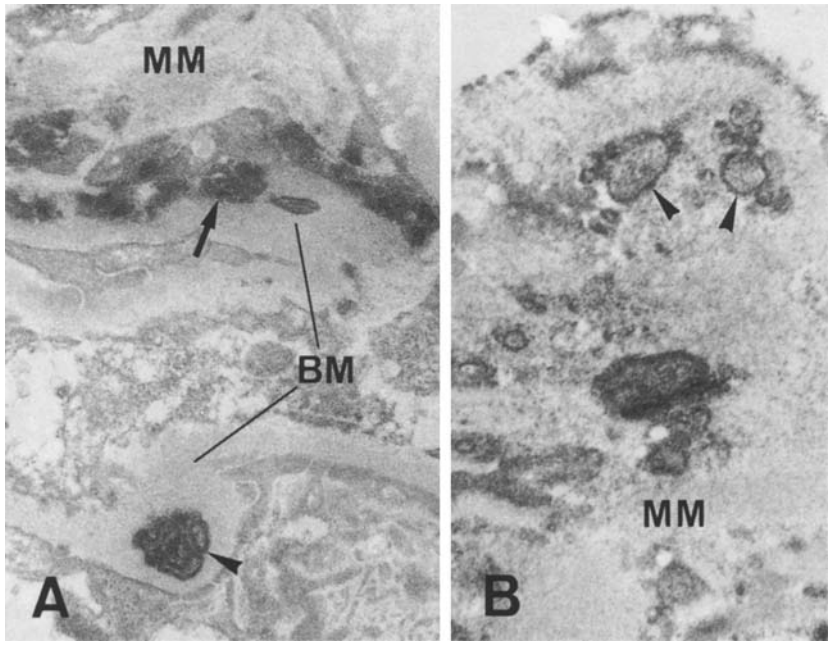

Fig. 5. Immunoelectron micrographs of glomeruli from patients with IgA nephropathy, stained for C3. Note homogeneous staining in the paramesangial zone (**A**, *arrow*) and staining associated with SMS in the GBM (**A** and **B**, *arrow heads*). Original magnification: **A** ×5000, and **B** ×10000

IgA or C3 in one patients. In the TBM, there was a homology of staining of Poly C9-MA and C5 or C9. C3 staining was only focal, and frequently dissociated with MAC. IgA or IgG was rarely observed in the TBM.

In the normal kidney immunoelectron microscopy on material from two individuals who were 43 and 50 years old, there were small amounts of reaction products with Poly C9-MA in the mesangial matrix and GBM. The products were generally associated with SMS (Fig. 2).

Kidney tissue from 15 of the 30 patients with IgA nephropathy was used for immunoperoxidase staining with Poly C9-MA, or antibodies to IgA, IgG, C3, C4, C5 and C9, and observed on an electron microscope. Staining with Poly C9-MA revealed substantial reaction products in the mesangial area of 11 patients, and along the glomerular capillary walls of five patients. In the mesangium, patterns and distributions in the MAC staining were various (Fig. 3A and B); (1) homogeneous fine-granular deposits between the GBM enclosing

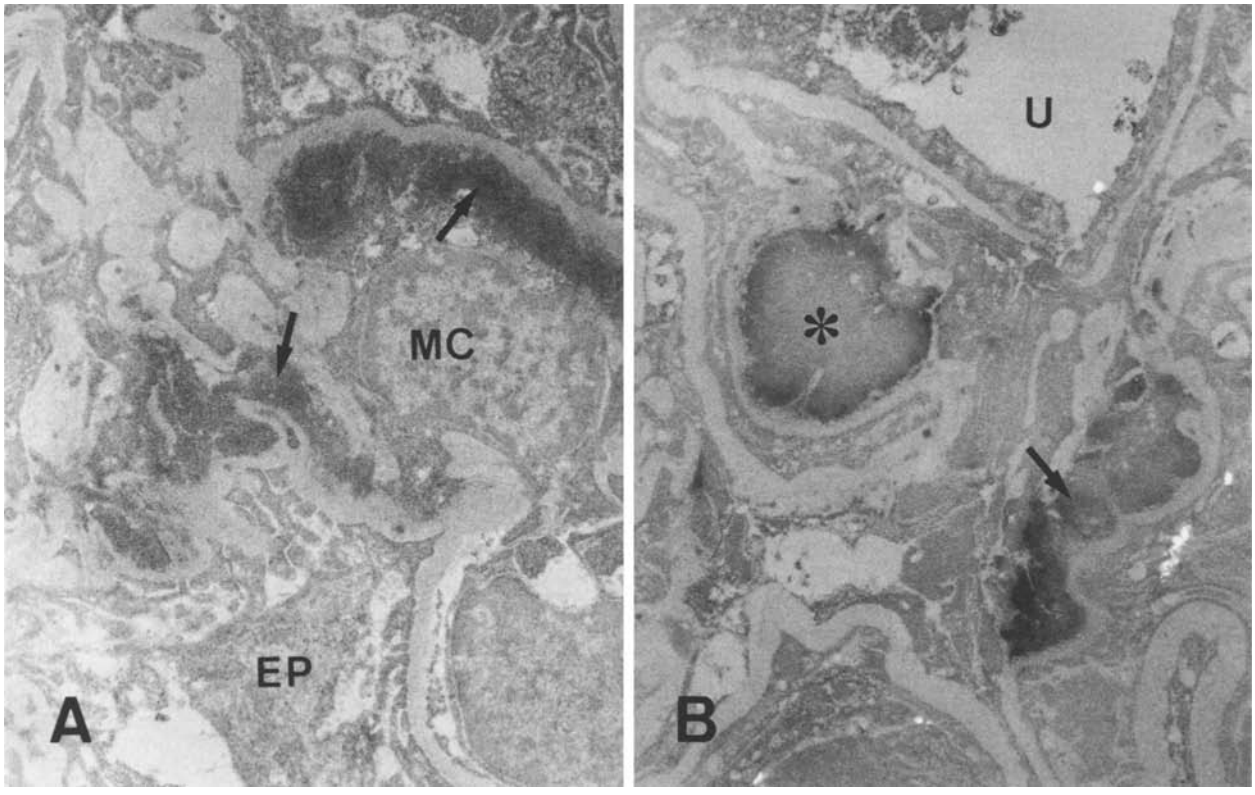

Fig. 6. Immunoelectron micrographs of glomeruli of IgA nephropathy, stained for C5 (**A**) and C9 (**B**). Note homogeneous staining in the paramesangial zone (**A** and **B**, *arrows*), and the staining of mesangial EDD (**B**, *asterisk*). Original magnification: **A** ×3500 and **B** ×3000

the mesangium and mesangial cells (paramesangial zone), (2) ring-shaped or ribbon-like staining associated with SMS, often in clusters, in the mesangial matrix, and (3) patchy staining within the mesangial EDD. MAC staining along the glomerular capillaries was observed as ring-shaped structures of various sizes (40–250 nm), or ribbon-like structures in the GBM matrix. Small amounts of MAC were present in the glomerular subepithelial space, which was also frequently associated with SMS (Fig. 3C). MAC was rarely observed in the subendothelial space. Characteristic cylindrical appearance of MAC was not observed anywhere at magnifications of up to ×50000–100000.

Direct peroxidase staining with anti-IgA antibody was positive in all 15 patients. Homogeneous IgA deposition was found predominantly beneath the GBM in the paramesangial zone and occasionally in the mesangial matrix, mesangial deposits, and glomerular subendothelial space (Fig. 4). IgG was positive in 10 patients; the staining was similar in distribution and pattern to IgA. C3, C5 and C9 were distributed beneath the mesangial BM, in the mesangial matrix, similarly to MAC, and in the entire or the periphery of mesangial EDD (Figs. 5 and 6). C3, C5 and C9 deposits in the mesangial and GBM matrices were frequently associated with SMS. Small C4 deposits were found in the mesangium of two of the 15 patients.

Staining with Poly C9-MA was evaluated in the TBM of the tissue from three patients. The reaction products showed ring-shaped structures, identical to those observed in the mesangial matrix and GBM (Fig. 7).

There was no relationship between immunofluorescence grading of glomerular MAC deposition and proteinuria, macroscopic haematuria, or duration of illness before renal biopsy (Fig. 8). Two patients with moderate renal failure showed 2+ positive MAC staining in the sclerosed area of the glomeruli. MAC staining was not related to the histological findings, i.e., glomerular crescents or focal accentuation. Tubular MAC staining failed to correlate with tubulo-interstitial changes on the light microscopy.

Discussion

In the present study we examined MAC deposition in the renal tissue of patients with IgA nephro-

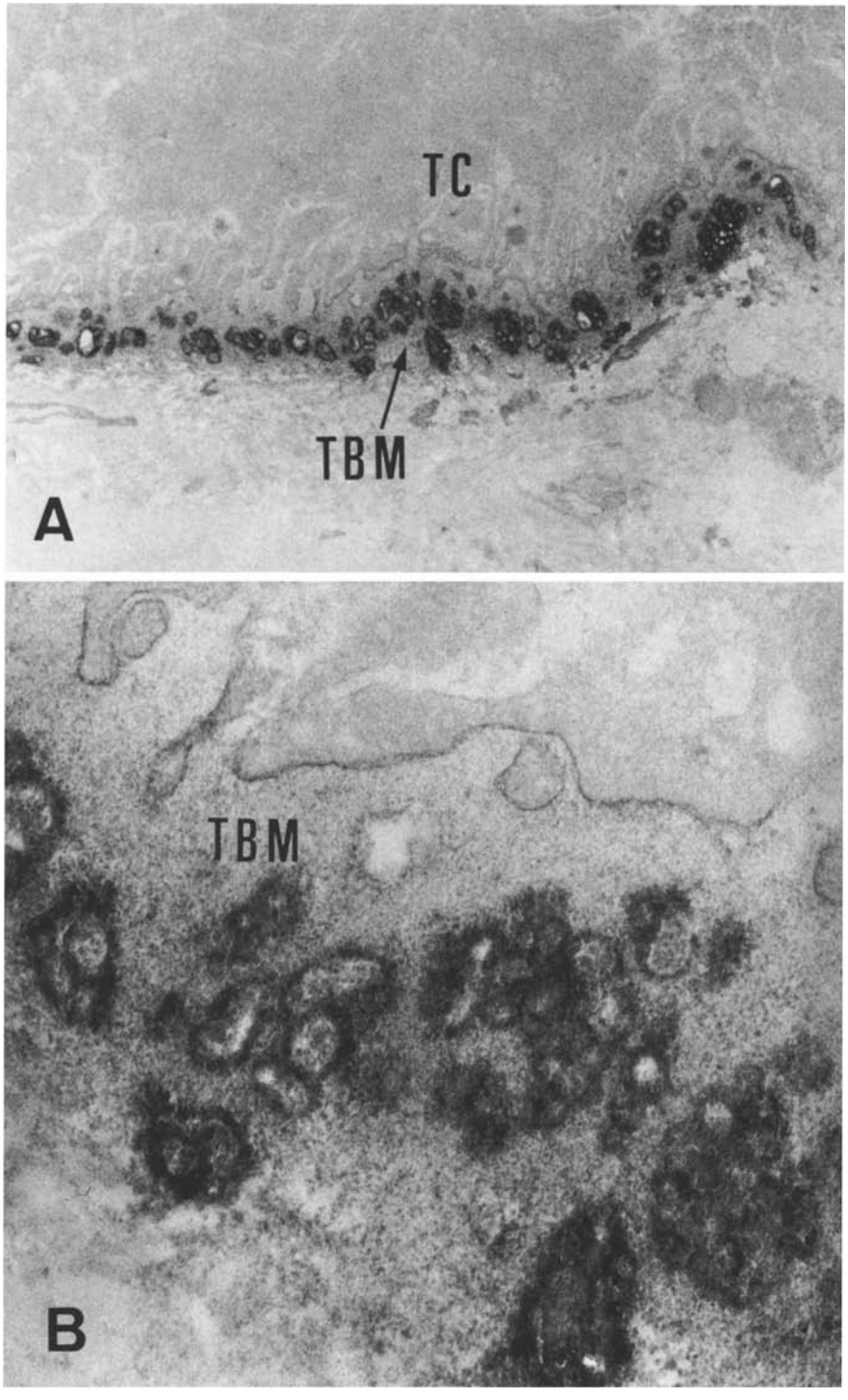

Fig. 7. Immunoelectron micrographs illustrating MAC staining along the TBM of IgA nephropathy. Note the intense labeling with ring-shaped structures. Original magnification: **A** ×3000, and **B** ×15000. *TC*: tubular epithelial cell. *TBM*: tubular basement membrane

pathy by immunofluorescent and immunoelectron microscopies using a well characterized monoclonal antibody to a neoantigen of C9 assembled in the MAC. In the normal kidney specimen, MAC was deposited along the TBM and blood vessel walls, and weakly in the mesangium, which is in agreement with the previous observations by other investigators (Falk et al. 1983; Hinglais et al. 1986). Significantly more MAC deposition, in both intensity and distribution, was found in the glomeruli of 22 of 30 patients with IgA nephropathy by immunofluorescence and 11 of 15 patients by

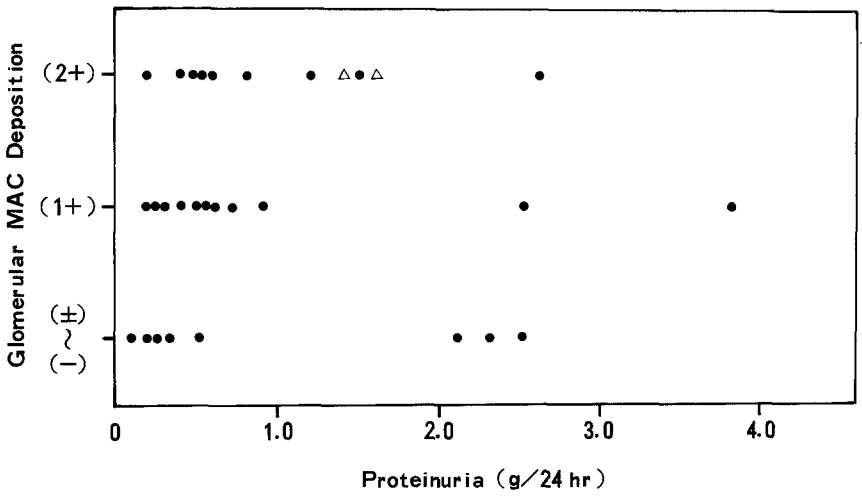

Fig. 8. Correlation between glomerular MAC deposition and proteinuria in patients with IgA nephropathy. ●: patient with creatinine clearance ≧70 ml/min/1.73 m^2. △: patient with creatinine clearance <70 ml/min/1.73 m^2

immunoelectron microscopy. Immunofluorescence localization of MAC, predominantly in the mesangium and occasionally along the glomerular capillary walls, was generally concordant with the deposition of IgA, IgG, C3, C5 and C9.

Several previous reports (Sinniah et al. 1981; Egido et al. 1983; Dysart et al. 1983) have shown that the paramesangial zone is the major site for localization of EDD and IgA deposits in the mesangium of IgA nephropathy. Immunoperoxidase staining revealed that the pattern and shape of MAC staining in this locus were analogous to those of IgA, and that complement components (C3, C5 and C9) were also localized in the paramesangial zone. In addition, MAC was detected within the mesangial EDD, where immunoglobulins (IgA and IgG) and complement components were also frequently stained. $C1_q$ and C4 were rarely present in the glomeruli of IgA nephropathy. As suggested previously (Egido et al. 1983), these data indicated that activation of complement with assembly of C5b–9 complexes occurs, mainly triggered by an alternative pathway mechanism, in the mesangium of IgA nephropathy, and is related to the paramesangial lesions and mesangial EDD. The association between immune deposits and MAC has already been reported in experimental immune complex diseases (Koffler et al. 1983; Adler et al. 1984; Perkinson et al. 1985) and several types of human renal diseases, including lupus nephritis, membranoproliferative glomerulonephritis and membranous nephropathy (Biesecker et al. 1981; Falk et al. 1983; Cosyns et al. 1986; Hinglais et al. 1986; Falk et al. 1987; Rauterberg et al. 1987; Falk et al. 1987).

In the matrix of mesangium, GBM and TBM, MAC was demonstrated as ring-shaped structures of various sizes (but definitely larger than the characteristic cyrindrical appearance of MAC) or ribbon-like configurations. These structures are quite similar to SMS reported by several investigators in normal and diseased human glomeruli (Nagel et al. 1969; Olson et al. 1974; Bariety and Callard 1975; Carlson et al. 1986; Cosyns et al. 1986; Hinglais et al. 1986), myocardium (Ferrans et al. 1976), seminiferous tubules (Salmon and Hedinger 1982), thyroids (Pfaltz and Hedinger 1986) and alveolar basement membrane (Okada M and Yoshioka K, unpublished observation). The origin of SMS is obscure, but they appear to be derived from cell membrane debris. The observations that heat-killed human kidney cells (Baker et al. 1984) or cytoskeletal intermediate filaments (Linder et al. 1979) can activate the complement system, and the close association between MAC deposition and antigen loss on the epithelial cells in the sclerosed glomeruli (Yoshioka et al. 1987b) suggest that MAC formation is mediated by cell remnants or SMS. As for the mechanism of MAC formation in glomerular SMS, in situ complement activation is more likely than entrapping of C5b–9 complexes from the circulation, since we found co-deposition of C3 and MAC in the SMS. We assume that MAC deposition in SMS is related to proliferation and turnover of the glomerular mesangial cells, because MAC deposits associated with SMS were significantly more abundant in the mesangial matrix of IgA nephropathy than in that of normal kidney. Although IgA deposits were localized in the subendothelial space of the glomerular capillary walls, MAC was rarely found in the locus. MAC deposits in the subepithelial space have also been frequently associated with SMS.

The pathogenic role of MAC for the develop-

ment of proteinuria has been demonstrated in an experimental model of membranous nephropathy in rabbits (Groggel et al. 1983). However, there was little relation between MAC deposition and proteinuria or other clinical findings of patients with IgA nephropathy, which is in contradiction with the findings by Rauterberg and coworkers (1987). MAC is known to be a stable structure which could remain in the tissue for a long time (Bhakdi and Traum-Jensen 1982). The MAC deposition we observed in the tissue might be a "past" event, which can be dissociated with "present" pathological or clinical findings. Long term follow-up might differentiate the prognosis of patients with glomerular MAC deposition from that of patients without it. However, some patients we studied were proteinuric without significant glomerular MAC deposition. Cosyns et al. (1986), who studied in situ complement activation in de novo membranous nephropathy, suggested that MAC formation was not required for the induction of proteinuria. There might be some mechanisms other than terminal complement system activation, such as limited complement activation at the C3 cleavage step (Cosyns et al. 1986), or coagulation and monocytes/macrophages sysetem (Takemura et al. 1987), which contribute more to the direct tissue injury in IgA nephropathy.

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