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Immunohistochemical Study of the Membrane Attack Complex of Complement and S-Protein in Idiopathic and Secondary Membranous Nephropathy

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Twenty-eight renal biopsies from 12 patients with idiopathic membranous nephropathy (MN), eight patients with lupus MN, and eight patients with hepatitis B virus-(HBV) related MN were investigated by immunofluorescence for the presence of C5b-C9 neoantigens of the terminal sequence of complement and for S-protein, which is a regulatory component of the membrane attack complex (MAC). Glomerular MAC was detected in 50% of patients with idiopathic MN, in 75% of patients with lupus MN, and in only 12.5% of the HBsAg carrier with MN. Glomerular adhesions to Bowman's capsule were associated with a high incidence of glomerular MAC deposition only in patients with idiopathic MN. Lupus patients had a high incidence of MAC deposition and patients with HBV-related MN had a low incidence of MAC deposition, in both cases regardless of the presence of glomerular capsular adhesions. It is unlikely that deposition of S-protein could inhibit the glomerular damage in idiopathic or lupus MN because significant glomerular capsular adhesions and MAC deposition were observed despite the concomitant glomerular deposition of S-protein. It was concluded that activation of terminal components of complement may play a role in glomerular injuries in idiopathic and lupus MN. The rare occurrence of glomerular MAC deposition in HBV-related MN could be related to its distinct immunopathogenetic mechanism and its indolent clinical course. (Am J Pathol 1989, 135: 469–476)

Membranous nephropathy (MN), a well-characterized glomerular disease, is the most common form of adult idiopathic nephrotic syndrome.[1] The disease is characterized by discontinuous deposits of immunoglobulin and complement developing exclusively in a subepithelial distribution along the outer surface of the glomerular capillary wall. These deposits induce a marked and nonselective increase in glomerular permeability to protein in the absence of any significant histologic evidence of inflammation.[2] Membranous nephropathy occurs in other systemic diseases, such as systemic lupus erythematosus and hepatitis B virus (HBV) infection, and in malignancy and as a result of drug medication. Nevertheless, a definite etiology is not found in a proportion of "idiopathic/primary" membranous nephropathy.

Although the etiology of primary MN is unknown, its ability to produce virtually identical glomerular lesions in animal models led to considerable insight into the immunologic mechanisms underlying this disease. Recently, Couser and associates[3] reported that the most likely mechanism of capillary wall injury is membrane damage induced by the formation of the C5b-C9 complex. S-protein is generally considered to be one of the regulatory components of membrane attack complex (MAC).[4]

The purpose of this study was to determine the pattern of deposition of C5b-C9, S-protein, or both, and to determine its clinico-pathologic correlation in primary membranous nephropathy and membranous nephropathy due to lupus erythematosus and chronic HBV infection.

Materials and Methods

Immunofluorescence

Percutaneous renal biopsy specimens were obtained from 28 patients with primary or secondary MN (eight pa-

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tients with lupus nephritis and eight patients with HBV-related MN) and from eight autopsy subjects without glomerular diseases. Light microscopic diagnosis of MN was made by diffuse thickening of the capillary basement membrane with spikes demonstrated by silver impregnation. Primary MN was diagnosed after exclusion of patients with systemic lupus erythematosus (SLE), infection (including syphilis, malaria, and HBV), diabetes mellitus, and malignancy, and of patients who were taking medication. The diagnosis of SLE was made if the patient fulfilled at one time at least four of the American Rheumatism Association 1982 revised criteria.[5] HBV-related MN was made when the patient demonstrated a persistence of hepatitis B surface antigen (HBsAg) and a high titer of antibody to hepatitis B core antigen (anti-HBc) in the serum.

Immunofluorescence was performed as previously described.[6] In brief, 3- to 4-μm thick cryostat sections were stained with fluorescein isothiocyanate (FITC)-labeled anti-human IgA, IgM, IgG, C1q, C4, or C3 antisera (Dakopatts, Denmark) (F/P molar ratios ranged from 1.8 to 2.9). These sections were also stained by indirect immunofluorescence with rabbit anti-human S-protein or C5b-C9 antisera (Behringwerke AG, FRG) and with FITC swine anti-rabbit IgG ($F(ab')_2$) antiserum (Dakopatts). Consecutive frozen sections from patients with HBV-related MN were also stained for HBsAg, hepatitis B core antigen (HBcAg), and hepatitis B e antigen (HBeAg) by direct immunofluorescence using a 1:10 dilution of murine monoclonal antibodies from the Jichi Medical School, Japan. The specificity and properties of these monoclonal antibodies have been previously reported.[7] The optimum incubation conditions for immunofluorescence were determined by preliminary experiments. The intensity of the fluorescence was graded independently by two observers (FML and STHL) without the knowledgement of previous clinical and pathologic findings and the nature of the antisera. Grades were assigned as follows: none (0), 1(+), 2(+), and 3(+).

Controlled renal biopsy samples included IgA nephropathy 5, mesangiocapillary glomerulonephritis 3, focal glomerulosclerosis 6, lipoid nephrosis 6, sclerosing glomerulonephritis 6, and autopsy specimens with no renal pathology 6.

Characterization of Rabbit Anti-human Antisera Against MAC and S-protein

Specificities of rabbit anti-human C5b-C9 (MAC) and S-protein antisera were determined in previous studies by immunodiffusion, immunoelectrophoresis, or both.[8] Monospecificity of the anti-MAC antiserum was further assessed in our laboratory by the Ouchterlony double immunodiffusion method. EDTA-added normal human plasma, zymosan-activated normal human serum, and purified human C5, C7-8, and C9 were used as antigens. Monospecificity of the anti-S-protein antiserum against S-protein but not the arg-gly-asp sequence was also assessed by the Ouchterlony double immunodiffusion method using C3b and zymosan-activated normal human serum as antigens.

Light Microscopy and Electron Microscopy

Renal biopsy specimens stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and periodic acid-methenamine silver (PASM) were examined by light microscopy. Glomerular damages were suggested by the presence of obsolescent glomeruli, segmental sclerosis, crescent formation, and capsular adhesion at one or more sites. At least eight consecutive sections were studied for each biopsy specimen to detect capsular adhesion at different sites. For transmission electron microscopy, tissue was fixed in phosphate-buffered 2.5% glutaraldehyde, postfixed in 1.5% osmium, embedded in Poly-Bed 812 (Polysciences, Inc.), stained with lead citrate and uranyl acetate, and examined with a Zeiss EM10C electron microscope.

Parameters of Disease Activity

The following laboratory investigations were performed at the time of renal biopsy: creatinine clearance, anti-nuclear factor, anti-DNA antibody, HBsAg, anti-HBc, HBeAg, blood urea nitrogen (BUN), serum creatinine, daily urinary protein, and serum IgA, IgG, IgM, C3 and C4.

Statistical Analysis

The Fisher's exact test and Rank-sum test were used for statistical analysis of clinical and pathologic data when appropriate.

Results

Monospecificity of Rabbit Anti-MAC and Anti-S-Protein Antisera

Using the Ouchterlony method, anti-MAC formed a single precipated band zymosan-activated normal human serum but not with the EDTA-added normal human plasma or individual complement component of the C5b-C9 com-

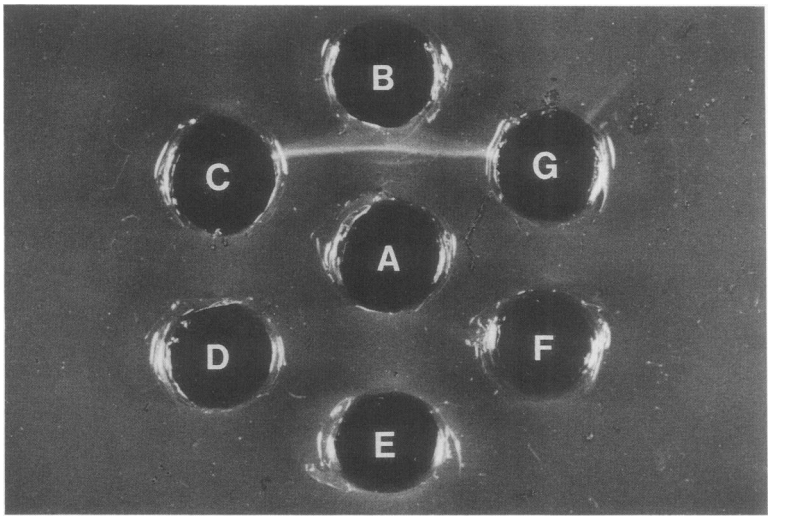

Figure 1. *Ouchterlony double immunodiffusion between rabbit anti-MAC (A) and zymosan-activated human serum (B), EDTA-added normal human plasma (C), C3b (D), C5 (E), C7-8 (F), and C9 (G). Anti-MAC showed a single precipitation band with zymosan-activated human serum.*

plex (Figure 1). The anti-S-protein did not precipitate with either purified human C3b or zymosan-activated normal human serum.

Immunofluorescence and Histopathologic Changes

IgG was the predominant immunoglobulin in glomeruli in all patients with primary MN (Table 1). Depositions of IgA were observed in one patient (8.3%) and of IgM in another (8.3%). C1q, C4, and C3 were observed in six (50%), 11 (92%), and two patients (16.7%), respectively. MAC and S-protein were observed in six (50%) and eight patients (66.7%), respectively. S-protein coexisted with MAC in six of the 12 patients (50%). The distribution of the immunoglobulins, complements, MAC, and S-protein was granular along the capillary basement membrane (Figure 2).

Polyclonal staining of immunoglobulins was demonstrated in glomeruli from patients with lupus MN (Table 1). C1q, C4, and C3 were observed in six (75%), eight (100%), and six patients (75%), respectively. MAC or S-protein was observed in six (75%) and seven patients (87.5%), respectively. S-protein coexisted with MAC in six of the eight patients (75%) (Figure 3). Similarly, the distribution of the immunoglobulins, complements, MAC, and S-protein was granular along the capillary basement membrane.

IgG was the predominant immunoglobulin in glomeruli in all patients with HBV-related MN (Table 1). Deposition of IgM was observed in one patient (12.5%) only. C1q, C3, and C4 were observed in two (25%), eight (100%), and three patients (37.5%), respectively. MAC and S-protein were observed in one (12.5%) and five patients (62.5%), respectively. S-protein coexisted with MAC in only one of eight patients (12.5%). The percentage of positive MAC staining in HBV-related MN was apparently less than that of primary MN ($P = 0.215$) and significantly less than that of lupus MN ($P = 0.043$). The distribution of complements, MAC, and S-protein was granular along the capillary basement membrane as observed with the immunoglobulin staining. Six of the eight biopsies (75%) showed granular capillary deposits of HBeAg by monoclonal antibodies, but HBV antigens were not detected in other non-HBsAg carriers. C1q, C3, C4, immunoglobulins, and HBV antigens were not found in renal biopsies from the autopsy controls. Sparse labeling of MAC and S-protein was occasionally seen in the mesangial areas in the controlled autopsy biopsies (Figure 4). In the other diseased controlled biopsies that lacked C5b-C9 deposits, capillary deposits of S-protein were observed in two of three biopsies with mesangiocapillary glomerulonephritis and in one of three biopsies with focal glomerulosclerosis. Mesangial depositions of S-protein were also detected in three of five biopsies with lipoid nephrosis, in one of two biopsies with sclerosing glomerulonephritis, and in two of two biopsies with IgA nephropathy.

Obsolescent glomeruli and segmental sclerosis were detected in only four and five renal biopsies, respectively. Glomerular adhesions to Bowman's capsule (synechias) were detected in 12 of the 28 renal biopsies. Five of the six biopsies with primary MN had glomerular MAC deposition in the presence of capsular adhesion, but only one of the six biopsies with primary MN had glomerular MAC deposition in the absence of capsular adhesion. Lupus patients had a high incidence of MAC deposition and patients with HBV-related MN had a low incidence of MAC deposition, both of which findings existed irrespective of the presence of glomerular capsular adhesions. Glomerular deposition of S-protein was detected in high percent-

Table 1. *Deposition of Immunoglobulins, Complement and S-Protein in Glomeruli from Patients with Membranous Nephropathy*

Case	IgG	IgM	IgA	C1q	C3	C4	C5b-C9 (MAC)	S-protein	HBsAg	HBcAg	HBeAg
Primary MN											
1	3	0	0	0	2	0	0	0	0	0	0
2	2	0	0	0	2	0	0	0	0	0	0
3	2	1	0	0	1	0	0	1	0	0	0
4	3	0	0	2	1	0	0	1	0	0	0
5	3	0	0	0	2	0	0	0	0	0	0
6	3	0	0	1	3	0	1	2	0	0	0
7	3	0	0	1	3	1	1	1	0	0	0
8	2	0	0	0	2	0	1	1	0	0	0
9	3	0	0	1	0	0	1	1	0	0	0
10	3	0	1	2	3	1	2	2	0	0	0
11	3	0	0	1	2	0	0	0	0	0	0
12	2	0	0	0	2	0	1	2	0	0	0
Lupus MN											
13	2	2	2	0	2	0	0	0	0	0	0
14	3	2	1	0	2	0	2	1	0	0	0
15	3	3	3	3	3	2	1	3	0	0	0
16	2	2	3	3	2	1	1	2	0	0	0
17	3	1	3	2	3	3	2	3	0	0	0
18	3	2	2	2	2	1	1	2	0	0	0
19	2	2	2	1	3	1	1	1	0	0	0
20	3	2	2	2	2	1	0	2	0	0	0
HBV MN											
21	3	1	0	0	2	0	0	0	0	0	2
22	3	0	0	0	1	0	0	0	0	0	0
23	3	0	0	2	2	3	0	2	0	0	3
24	2	0	0	0	2	1	0	1	0	0	1
25	3	0	0	0	2	0	0	1	0	0	2
26	3	0	0	0	2	0	0	2	0	0	2
27	3	0	0	1	2	1	1	2	0	0	2
28	2	0	0	0	2	0	0	0	0	0	0

Immunofluorescence was scored as 0, 1+, 2+, and 3+.

ages of patients with primary and secondary MN regardless of whether or not glomerular capsular adhesions were present. Significant crescent formation (involving more than 50% of glomeruli) occurred in only one patient with primary MN (case 12) and in two patients with lupus MN (cases 14 and 17). Glomerular deposition of both MAC and S-protein was detected in all three biopsies.

Clinical Parameters

There was no significant correlation among the serum concentration of blood urea nitrogen, creatinine, C3, or C4, the deposition of immunoglobulins, and the deposition of MAC or S-protein in the three groups of patients with MN. The number of patients in individual groups of MN was too small for statistical analysis of correlation between glomerular MAC/S-protein deposition and renal function. However, when all patients with MN were considered as a single group, the daily urinary loss of protein was significantly higher in patients with glomerular MAC deposits (N = 13) than in those without glomerular MAC deposits (N = 15) (3.6 ± 1.4 g/l *versus* 2.3 + 1.1 g/l; $P < 0.02$). Similar findings were not observed with S-protein deposition.

Discussion

Data from human and experimental MN strongly suggest *in situ* immune complex formation plays an important role in the immunopathogenesis of primary MN.[9] Circulating immune complexes have been detected in a minority of patients in some studies of MN in human, but have not been found in significant quantity in others.[10] Membranous lupus nephritis is characterized by polyclonal immunoglobulin deposition in the glomeruli. Although DNA-anti-DNA complexes are present in glomeruli during lupus diseases, the mechanism by which they accumulate in kidneys remains unknown. Still, the possibility exists that deposition of circulating complexes and formation *in situ* might be simultaneously or successively involved in the same patient, or that these different mechanisms might be at work in different patients.[11] Contrary to these findings, circulating immune complexes have been sug-

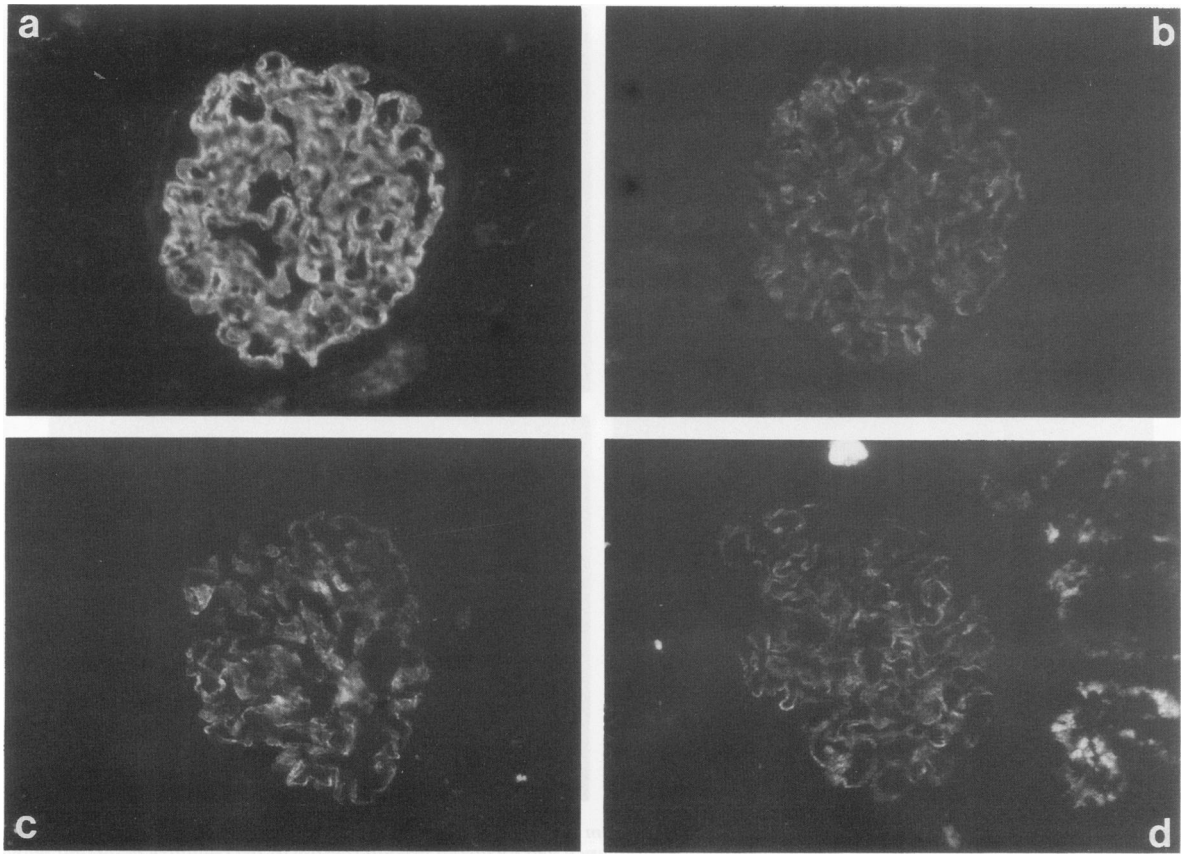

Figure 2. *Immunofluorescent micrographs of kidney sections from case 7 with idiopathic membranous nephropathy stained for C3* (a), *C4* (b), *MAC* (c), *and S-protein* (d). *The granular immunofluorescence was localized predominantly in the capillary basement membrane (original magnification × 160).*

gested as playing a pathogenetic role in HBV related MN.[12,13] Therefore, despite the apparent homogeneity of the light microscopy finding in these MN, their immunopathogenesis appears to be different.

Glomerular injury in MN has recently been shown to be mediated through the formation of the C5b-C9 membrane attack complex (MAC) of the complement system by the immune deposits.[14,15] The MAC of complement represents a multimolecular assembly that involves C5, C6, C7, C8, and C9.[4,16] S-protein has dual functions as it prevents innocent bystander lysis by binding to fluid phase C5b-C7 or C5b-C8, and it also reduces C9 polymerization in the C5b-C9 complex.[17] Experimentally, the glomerular injury in MN can be completely abrogated by any maneuver that prevents formation of the MAC of the complement system.[3] The deposition of MAC has been suggested to be related to crescent formation and capsular adhesion in nephropathies such as IgA nephropathy,[7] although contradictory data have also been reported.[18]

The purpose of the present study was to determine whether or not different immunopathogenetic mechanisms in primary and secondary membranous nephropathy could alter the glomerular deposition of MAC, S-protein, or both and to determine the clinico-pathologic significance of such depositions. Glomerular capillary deposition of C5b-C9 neoantigen has previously been reported in six patients with idiopathic MN,[15] two patients with drug-induced MN,[15] eight patients with de novo MN who underwent transplants,[19] and two patients with lupus MN.[16] *In situ* activation of complement, rather than trapping of soluble C5b-C9 complexes, occurs in the glomeruli as the coexistence of C3c, C3d, and H antigen is strongly suggestive of *in situ* C3 cleavage.[16,19] It has also been suggested that *in situ* activation of the whole complement sequence throughout C5b-C9 occurs only on large immune deposits with more advanced MN.[19] Glomerular MAC was detected in 50% of our patients with idiopathic MN, and the percentage was similar to that of *de novo* MN in patients who underwent renal transplant.[19] MAC deposition was apparently associated with the presence of glomerular capsular adhesion in idiopathic MN, although the number of patients was too small for statistical analysis. Glomerular deposits of MAC were detected in 75% of biopsies from patients with lupus MN, and the

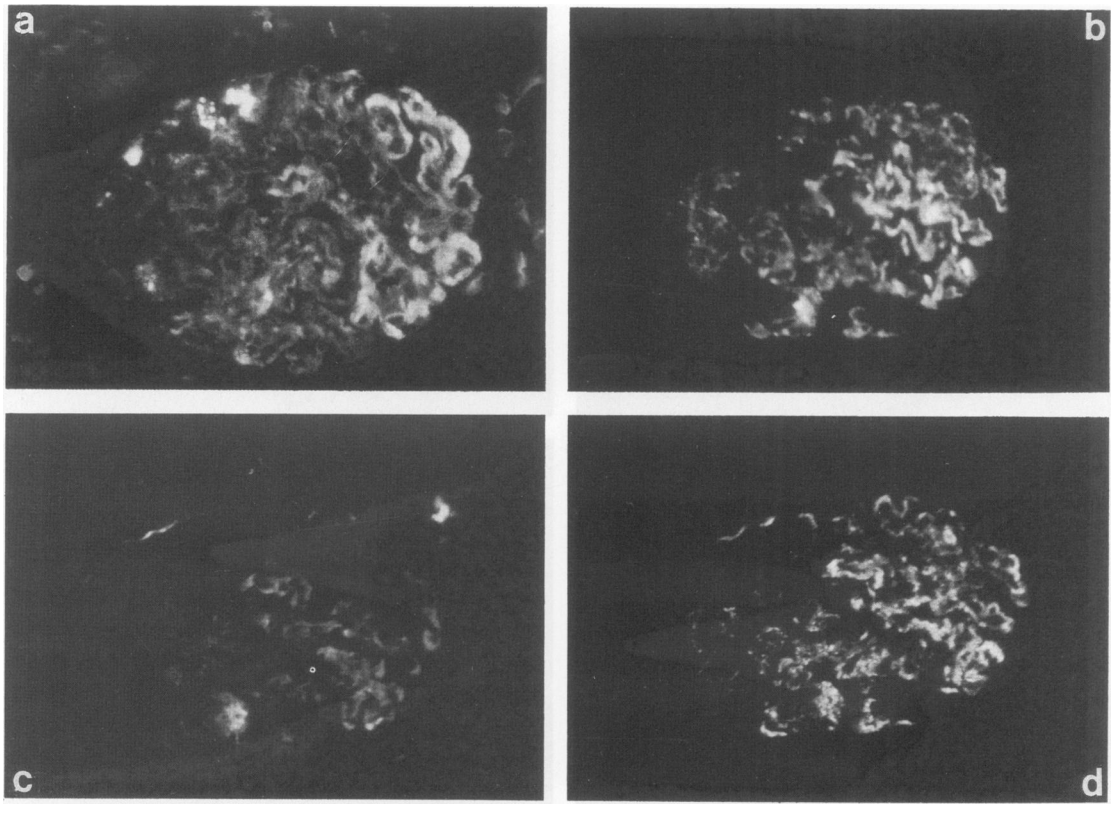

Figure 3. *Immunofluorescent micrographs of kidney sections from case 17 with lupus membranous nephropathy stained for C3* (a), *C4* (b), *MAC* (c), *and S-protein* (d). *The granular immunofluorescence was localized predominantly in the capillary basement membrane* a, *(original magnification × 180; b–d, original magnification × 160).*

percentage was similar to the 67% obtained from three lupus patients studied by Biesecker and associates.[16] Contrary to the findings in idiopathic MN, MAC deposition was observed in most cases of lupus MN irrespective of the presence of significant glomerular capsular adhesion. Whereas immune complex formation *in situ* apparently play an important pathogenetic role in idiopathic and lupus MN, HBV-related MN is believed to be related to glomerular trapping of circulating immune complexes.[12,13] In our study, we confirmed that HBeAg is the sole HBV antigen associated with HBV-related MN, as was previously suggested by Ito and associate.[8] Contrary to the findings in idiopathic MN and lupus MN, glomerular MAC deposition was detected in only 12.5% of biopsies from these HBsAg carriers with MN. If glomerular capillary injury in MN is mediated through the C5b-C9 complex formation,[3] the low incidence of glomerular MAC deposition may indicate a more benign clinical course for HBV-related MN when compared with idiopathic or lupus MN. This hypothesis is supported by the clinical observation that the nephrotic syndrome associated with HBV-related MN tends to regress spontaneously without therapy in 30% to 60% of these patients.[8,20] Conclusions regarding the pathologic correlation between MAC deposition and crescent formation are difficult to make as crescent formation is uncommon in MN. Shulze and coworkers[21] suggested that urinary excretion of C5b-C9 occurs in early (and perhaps more active) cases of MN with more severe proteinuria and well-preserved renal function. Although a good correlation between renal function and glomerular MAC deposition was not demonstrated in our patients, those patients with glomerular MAC deposition did have a significantly heavier proteinuria than those without glomerular MAC deposition. These findings tend to support the pathogenetic role of MAC in MN.

Deposition of S-protein in glomeruli coexisted with that of MAC in 50% of idiopathic MN, in 75% of lupus MN, and in only 12.5% of HBV-related MN. The clinical significance of glomerular deposition of S-protein is still unknown. In addition to its ability to "neutralize" C5b-C9 in the fluid phase, S-protein (vitronectin) is one of several cell-attachment factors that bear the arg-gly-asp sequence. Nonspecific binding of anti-S-protein antiserum to exposed matrix receptors on damaged cell membranes is not a finding supported by the negative immunodiffusion test against C3b that contains the arg-gly-asp sequence. Its

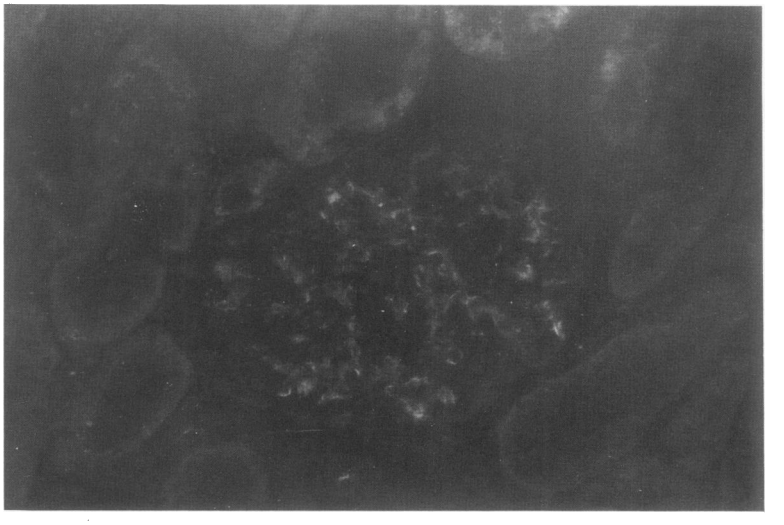

Figure 4. *Immunofluorescent micrograph of kidney section from autopsy-controlled biopsy with no renal pathology. No or sparse mesangial deposits of MAC were detected in these "normal" controlled samples (original magnification ×160)*

presence in tissue could potentially reflect binding to C5b-C9 that was shed from cell membrane or nonspecific entrappment by C5b-C9. These possibilities are considered less likely because S-protein is also present in some biopsies that lack C5b-C9 deposition. Glomerular S-protein deposition was reported to be associated with capsular adhesion and crescent formation in IgA nephropathy. Such correlation was not observed in our patients with MN. Although S-protein plays a regulatory role in MAC formation,[17] it is unlikely that deposition of S-protein inhibits the glomerular damage in idiopathic or lupus MN because significant glomerular capsular adhesions and MAC deposition were observed in such patients despite the concomitant glomerular deposition of S-protein.

In conclusion, the complement activation may play a role in glomerular injuries in idiopathic and lupus MN. The rarity of glomerular MAC deposition in HBV-related MN may be related to its distinct immunopathogenetic mechanism and its indolent clinical course.

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