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Immunoelectron microscopic localization of membrane attack complex and hepatitis B e antigen in membranous nephropathy

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Summary. Immunoelectron microscopy was used to localize membrane attack complex (MAC) and hepatitis B e (HBe) antigen in renal tissue specimens from a total of 9 patients with membranous nephropathy (MN); 6 with MN associated with a hepatitis B virus (HBV) infection, 2 with idiopathic MN, and 1 with lupus nephritis. All the patients were proteinuric, and 2 patients were classified as stage I–II, 6 as stage II, and 1 as stage IV. MAC, along with IgG and C3, was distributed within the subepithelial electron dense deposits in all the stages. MAC was also stained in the striated membranous structures within the glomerular basement membrane and mesangial matrix of some patients. In HBV-associated MN, HBe antigen was localized in the subepithelial electron dense deposits of 5 patients, while it was absent from the subepithelial deposits in a patient that was sero-positive for hepatitis B s antigen but negative for HBe antigen. This patient also lacked MAC deposition in these loci. These results suggest that MAC is associated with the formation of subepithelial deposits and proteinuria in MN. In HBV-associated MN, HBe antigen-antibody immune complex makes up the subepithelial deposits and is likely to activate the terminal components of complement *in situ*.

Key words: Membranous nephropathy – Membrane attack complex – Hepatitis B virus – Hepatitis B e antigen

Introduction

Membranous nephropathy (MN) is a chronic glomerular disease, characterized histologically by a thickening of the glomerular capillary walls and extramembranous deposits with only minor proliferative changes of the glomeruli. MN occurs in an idiopathic form, and is also associated with infections, exposure to heavy metals, systemic lupus erythematosus (SLE), diabetes mellitis, neoplasms, and renal vein thrombosis. MN is a common form of glomerulonephritis in Japanese children with persistant hepatitis B virus (HBV) infections (Takekoshi and Tanaka 1987). Evidence indicates that, among the 3 discrete HBV-associated antigens, HBs, HBc and HBe, HBe is most likely to be responsible for inducing MN (Takekoshi et al. 1979; Ito et al. 1981; Hirose et al. 1984).

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 disease (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; Miyamoto et al. 1988). MAC is formed after cleavage of the C5 by classical or alternative complement pathway mechanisms. In both normal and diseased kidney tissues, MAC is frequently localized in the glomerular basement membrane and mesangial matrix, and associated with the striated membranous structures by immunoelectron microscopy (Hinglais et al. 1986; Falk et al. 1987; Miyamoto et al. 1988). Studies of experimental MN induced by the immunization of normal and C6-deficient rabbits with cationic bovine serum albumin suggested that MAC is responsible for tissue damage and proteinuria (Groggel et al. 1983). However, the role of MAC in human MN is still unclear.

Using immunoelectron microscopy, we examined the localization of MAC and HBe antigen in kidney tissue sections from patients with MN

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of unknown etiology and in that secondary to HBV infection or SLE.

Materials and methods

Tissue samples consisted of routine renal biopsy specimens taken from 6 patients with HBV-associated MN, 2 with idiopathic MN, and 1 with MN associated with SLE (Table 1). The diagnosis was made based on clinical, light microscopic, immunofluorescent, and conventional electron microscopic findings. All patients were proteinuric. Renal function was impaired in 2 patients (patients 8 and 9). All 6 patients with HBV-associated MN were sero-positive for HBs antigen. HBe antigen was positive in the serum of 5 patients, and HBe antibody was positive in the remainder (patient 4). The stages of MN were graded by conventional electron microscopy according to the classification of Ehrenreich and Churg (1968).

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 antibodies were purchased from commercial sources: goat anti-human IgG (Cappel, PA, USA), goat anti-human C3 (Cappel), mouse monoclonal antibody to HBe antigen (Immunis, Tokyo, Japan), mouse monoclonal antibody to HBs antigen (Immunis), and goat anti-mouse IgG labeled with horse-radish peroxidase (HRP) (Cappel). The Fab' fragment of goat antisera to anti-human IgG or C3 was obtained by digestion with pepsin, followed by Sephadex G-200 (Pharmacia, Uppsala, Sweden) gel filtration (Miyamoto et al. 1988). HRP was conjugated by the periodate oxidation method (Nakane and Kawaoi 1974).

The dilution of the monoclonal antibody to HBs antigen or HBe antigen was determined by the indirect immunofluorescent staining of liver tissues obtained from a patient with liver cancer who was sero-positive for both HBs and HBe antigens.

Conventional electron microscopy was performed on samples fixed in 4% glutaraldehyde and osmium, and examined with a Hitachi H-800 electron microscope. One or two glomeruli from each specimen were examined. Immunoelectron microscopy was performed as previously described (Takemura et al. 1987; Miyamoto et al. 1988). In brief, the tissue, fixed for 4 h at 4° C in 2% paraformaldehyde-0.075 M lysine-0.01 M periodate fixative of McLean and Nakane (1974), was cut into 4 μm sections on a cryostat, and then treated with periodate and sodium borohydroxide to inhibit endogenous peroxidase (Isobe et al. 1977). The section was incubated at 4° C overnight with HRP-labeled Fab' fragments of anti-human IgG or C3, or the monoclonal antibody to MAC, HBe antigen or HBs antigen. In staining for MAC, HBe antigen and HBs antigen, the section was then incubated with normal human plasma-preabsorbed goat anti-mouse IgG labeled with HRP at room temperature for 45 min. After washing, all the sections were processed in a substrate solution, diaminobenzidine with 0.01% hydrogen peroxide. The tissues were fixed in osmic acid, and then embedded in Epon 812. Ultra-thin sections were cut and examined with a Hitachi H-800 electron microscope.

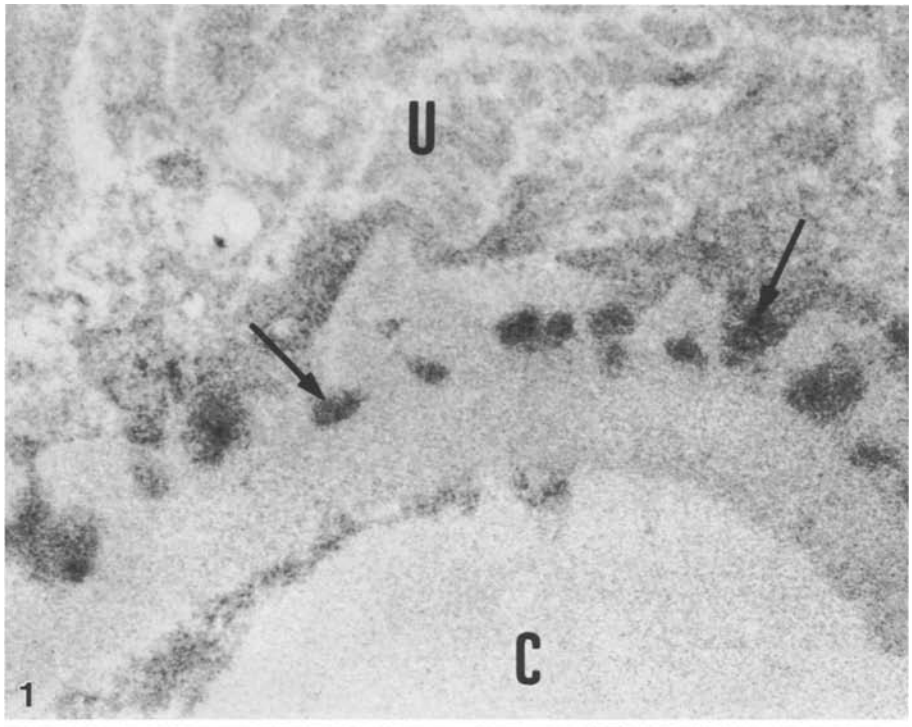
Results

Immuno-peroxidase staining revealed that MAC was distributed within the subepithelial electron dense deposits in 5 of 6 patients with HBV-associated MN, and all 3 patients with other types of MN (Table 1, Fig. 1 and 2). The deposits were unevenly stained for MAC in all the stages (stages I–II, II and IV) of MN we observed. MAC was also present as ring-shaped or ribbon-like structures in the glomerular basement membrane and mesangial matrix, which was associated with the striated membranous structures (Fig. 2). The MAC staining associated with these structures was more frequently seen in stage IV MN than stage I–II or stage II MN. Staining for IgG was intense and

Table 1. Immunoelectron microscopic observation of the glomeruli of patients with MN

Patient No.	Age	Sex	Serum HBeAg/HBsAg	Proteinuria (g/24 hr)	Stage	Deposition in the subepithelial EDD				
						MAC	IgG	C3	HBeAg	HBsAg
HBV-associated										
1	5	F	+/+	1.3	II	+	+	+	+	−
2	3	F	+/+	2.6	II	+	+	+	+	+
3	3	M	+/+	3.2	II	+	+	+	+	+
4*	13	M	−/+	1.5	II	−	+	+	−	−
5	8	F	+/+	0.7	II	+	+	+	+	−
6	4	F	+/+	3.8	II	+	+	+	+	−
Idiopathic										
7	5	M	−/−	2.4	I–II	+	+	+	n.d.	n.d.
8	15	M	−/−	9.8	IV	+	+	+	n.d.	n.d.
SLE										
9	18	F	−/−	6.0	I–II	+	n.d.	+	n.d.	n.d.

MN, membranous nephropathy; *EDD*, electron dense deposit; *HBeAg*, hepatitis B e antigen; *HBsAg*, hepatitis B s antigen; *MAC*, membrane attack complex; *HBV*, hepatitis B virus; *n.d.*, not done; *SLE*, systemic lupus erythematosus
* Sero-positive for HBe antibody



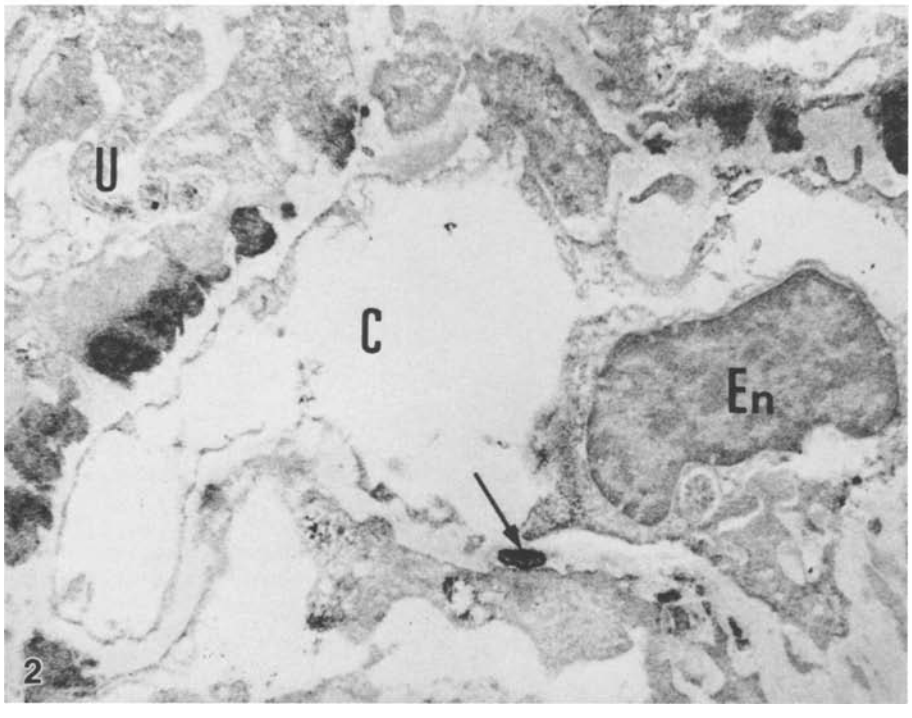


Fig. 1. Immunoelectron micrograph of a portion of glomeruli from a patient with SLE-associated MN (stage I–II), stained for localization of MAC. Note positive staining for MAC within subepithelial EDD (*arrows*). Original magnification: ×3000, *U*: urinary space; *C*: capillary lumen

Fig. 2. Immunoelectron micrograph of a portion of glomeruli from a patient with HBV-associated MN (stage II). Note uneven staining of MAC within the subepithelial EDD, and MAC associated with SMS in the glomerular basement membrane (*arrow*). Original magnification: ×3000, *En*: endothelial cell

homogeneous within the subepithelial deposits in all the patients. C3 was similarly present in all the patients (Fig. 3).

Positive staining for HBe antigen was observed in the subepithelial electron dense deposits in the glomeruli of 5 of 6 patients with HBV-associated MN (Table 1, Fig. 4). HBe antigen was absent from the glomeruli in 1 patient, who was seronegative for the HBe antigen. The patient also lacked MAC deposition in the glomeruli. Weak staining for the HBs antigen was found in the subepithelial deposits in 2 patients.

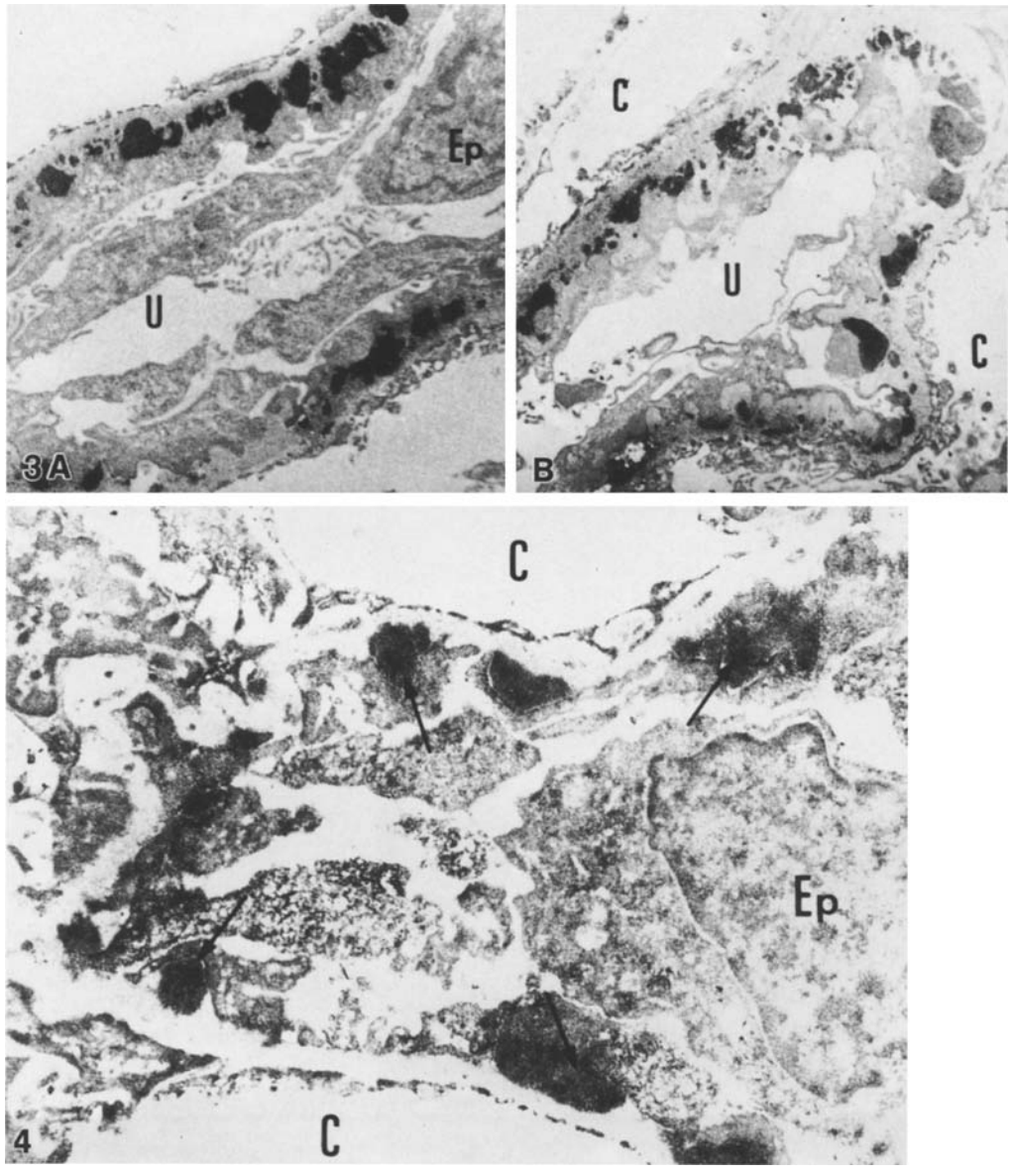


Fig. 3. Homogeneous staining for IgG (**A**) and C3 (**B**) in the subepithelial EDD in a patient with HBV-associated MN (stage II). Original magnification: A and B, × 2500, *Ep:* epithelial cell

Fig. 4. Electron micrograph of capillary walls in a glomerulus from patient with HBV-associated MN, stained by immunoperoxidase with a monoclonal antibody to HBe antigen. Note positive staining in the subepithelial EDD (*arrows*) Original magnification: × 3500

Discussion

The important contribution of the complement system to the pathogenesis of an experimental model of MN has been well accepted; complement depletion, achieved by the administration of cobra venom factor prevented the onset of proteinuria in passive Heymann's nephritis (Perkinson et al. 1985). Studies by Salant et al. (1980) and Groggel and co-workers (1983) have further suggested that complement-dependent glomerular capillary wall injury is attributed to the activation of terminal complement components, MAC, which can function independently of inflammatory cells. However, the role of MAC in the induction of proteinuria in human MN is still unclear.

In the present study, we demonstrated MAC deposition in the glomeruli of patients with idiopathic, HBV-associated and SLE-associated MN. In all the patients but one, MAC, along with IgG

and C3, were localized in the subepithelial electron dense deposits. MAC was positive in stage I–II, II and IV MN as classified by electron microscopic examination. This observation contradicts the report by Cosyns et al. (1986), who studied MAC deposition in kidney transplanted patients with *de novo* type MN, and found that, in 6 of 7 renal biopsies classified as stage I, immunofluorescent staining for MAC was restricted to the mesangial areas, and absent from the glomerular capillary walls. Immunoelectron microscopy was not performed in their studies, however. Our present studies suggest that MAC deposition is associated with an immune complex formation in the subepithelial area, and plays a nephritogenic role in mediating glomerular capillary wall injury and proteinuria, similar to that demonstrated in the animal models.

Within the matrix of the mesangium and glomerular basement membrane, MAC was frequently observed as ring-shaped structures or ribbon-like configurations. As reported previously (Hinglais et al. 1986; Falk et al. 1987; Miyamoto et al. 1988), they were apparently associated with the striated membranous structures. The origin of these structures is still obscure, but they appear to be derived from cell membrane. The role of the MAC in this locus is unclear, although the possibility of local activation of the complement system by damaged renal cells has been suggested (Yoshioka et al. 1987).

HBV infection provokes hepatic as well as extrahepatic manifestations. The association between HBV infection and MN, first described by Combes et al. (1971), has well been established particularly in areas with a high prevalence of HBV infection such as in East Asia and Africa. Using fluorescent antibody techniques, the deposition of HBe, HBs or HBc antigen was solely or concomitantly found in glomerular immune deposits in patients with MN (Ozawa et al. 1976; Takekoshi et al. 1979; Slusarczyk et al. 1980; Ito et al. 1981; Hirose et al. 1984). Although there is some disagreement about the pathogenic antigen, it is most likely that the HBe antigen is responsible for the induction of proteinuria in patients with HBV infections and MN, on the basis of the immunofluorescent findings and molecular size of the HBe antigen or IgG-bound HBe antigen (Takekoshi and Tanaka 1987).

Using immunoelectron microscopy we have confirmed the results of previous immunofluorescent studies by several investigators (Takekoshi et al. 1979; Ito et al. 1981; Hirose et al. 1984; Yoshikawa et al. 1985). HBe antigen was present in the subepithelial electron dense deposits in 5 of 6 patients with HBV-associated MN. Weak staining for the HBs antigen was also observed in the subepithelial deposits in 2 patients. Several authors (Ozawa et al. 1976; Cogan et al. 1977; Nagy et al. 1979; Slusarczyk et al. 1980; Ito et al. 1981; Magil et al. 1986) reported the deposition of HBs antigen in the glomeruli, which may reflect the entrapment of small fragments of HBs antigen in deposits separate from the circulation.

In summary, this study shows the important contribution of MAC and HBe antigen to the formation of immune deposits in the subepithelial area, and in the induction of proteinuria in patients with MN.

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