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Immunohistochemical localization of C3d fragment of complement and S-protein (vitronectin) in normal and diseased human kidneys: association with the C5b-9 complex and vitronectin receptor

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Abstract. The localization of C3d, a fragment produced by C3 activation and S-protein (vitronectin), a regulatory factor of C5b-9, was studied immunohistochemically in normal human kidney and renal biopsies from patients with several types of glomerulonephritis. Immunofluorescent staining of the normal kidneys showed that C3d was present along the glomerular basement membrane (GBM), tubular basement membrane (TBM) and arterioles, and that S-protein was present in the GBM, mesangium, TBM, and arterioles. Immunoelectron microscopy of isolated basement membranes showed that C3d was localized exclusively on the epithelial side of the GBM, and that S-protein was present along both the epithelial and endothelial sides. In nephritic tissues, glomerular staining of C3d, C5b-9, and S-protein was increased when compared with that in normal tissues. S-protein, frequently co-localized with C3d and C5b-9 neoantigen, was intensely positive in the immune deposits of glomerular capillaries and the mesangial area, overlapping the background staining of GBM and mesangial matrix. S-protein and its receptor were occasionally co-localized in the glomeruli. These findings indicate that C3d and S-protein are normally present in the glomeruli. Co-staining of C3d, C5b-9 neoantigen, and S-protein within the immune deposits of nephritic kidneys suggests in situ binding of S-protein to locally-formed C5b-9 complex, or merely co-distribution of S-protein with the complex, rather than trapping of large molecular SC5b-9 complex from the circulation.

Key words: Complement – S-protein – Glomerulonephritis – Vitronectin receptor

Introduction

Activation of the complement system through C3 can occur by the classical or the alternate pathway, and leads to the release of several biologically-active factors during the assembly of a common terminal pathway, C5b-9. The complement system is known to be an important mediator of glomerular injury in certain types of experimental nephritis, as well as in human glomerulonephritis (Couser et al. 1985). The activation of C3 results in the generation of C3a and C3b. C3b is further cleaved into small fragments, C3c (130 kDa) and C3d (40 kDa). Most C3d remains in the site where C3 is activated, but C3c does not. Therefore, detection of C3d indicates in situ complement activation (Lachman et al. 1982; Cosyns et al. 1986).

C5b-9 complex is found as two different forms, a membrane-bound form, membrane attack complex (MAC), and a fluid-phase, S-protein bound form, SC5b-9 (Podack 1988). The former is biologically active, and can cause cell membrane damage, such as lysis of red blood cell membranes or injury to the glomerular epithelial cell membrane. C5b-9 deposits have been observed in several types of experimental nephritis and are associated with the appearance of proteinuria (Couser et al. 1985). Studies of human glomerulonephritis have demonstrated C5b-9 within immune deposits (Falk et al. 1983; Hinglais et al. 1986; Miyamoto et al. 1988). The complex is also found in normal kidney tissue and in kidneys with non-nephritic conditions such as diabetes and obstructive uropathy (Falk et al. 1983; Hinglais et al. 1986). The deposits appear to be associated with the striated membranous structures present within the glomerular basement membrane (GBM) and tubular basement membrane (TBM) (Hinglais et al. 1986; Miyamoto et al. 1988). The role of C5b-9 in human nephritis is still unclear, since S-protein (vitronectin) is frequently co-stained with C5b-9 in human kidney tissues (Falk et al. 1987; Bariety et al. 1989). S-protein binds to C5b-8 or C5b-9 in the circulation, preventing C5b-9 from forming MAC and from lysing of target membranes. Recent evidence suggests that S-protein may also interact with the membrane-associated C5b-9 complex (Bhakdi et al. 1988). Sequence analysis of S-protein indicates that it is identical to vitronectin, the classical cell attachment

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and spreading factor (Junne and Stanley 1985). The incorporation of S-protein in the C5b-9 complex suggests that deposition of terminal complement complexes would form sites for cell adhesion. S-protein-C5b-9 complex has been shown to mediate cell adhesion through an integrin vitronectin receptor (Biesecker 1990).

In the present study, the deposition of C3d and S-protein was immunohistochemically demonstrated in biopsied renal tissues. The relationship of these proteins and C5b-9 and vitronectin receptor was also investigated in various types of human glomerulonephritis.

Materials and methods

The glomeruli and tubules were isolated separately from accident victims, by a sieving technique, as described previously (Yoshioka et al. 1986, 1988). The GBM was prepared from the isolated materials by ultrasonic disruption in 1 M sodium chloride/proteinase inhibitors or by detergent solubilization using 4% sodium deoxycholate. The distinction between the epithelial and endothelial sides of the GBM was made according to the findings of Williams et al. (1984): the denser and smoother side was taken as the epithelial side.

Renal biopsy specimens obtained from a total of 73 patients with the following diagnoses were used: IgA nephritis (22 patients), mesangial proliferative (non-IgA) glomerulonephritis (17), minimal change nephrotic syndrome (10), Henoch-Schonlein purpura nephritis (7), membranoproliferative glomerulonephritis (4; 1 of type I, 2 of type II, and 1 of type III), focal glomerular sclerosis (4), lupus nephritis (WHO class IV, 4), idiopathic membranous nephropathy (2), haemolytic uraemic syndrome (2), reflux nephropathy (1), rapidly progressive glomerulonephritis (1), and oligomeganephronia (1). A part of each tissue was fixed with 10% buffered-formalin and embedded in paraffin. Thin sections were stained with haematoxylin and eosin, PAS, and methenamine-silver. The increase in the mesangial matrix was graded as mild, moderate, and marked, as described previously (Yoshioka et al. 1989).

Histologically normal portions of kidney tissues from patients who underwent nephrectomy due to renal tumour or renal trauma were served as the normal controls.

The following monoclonal and polyclonal antibodies against complement components, S-protein, and vitronectin receptor were used in this study: human C3d: monoclonal antibody (mouse IgG_1, Chemicon), polyclonal antibody (sheep antisera, Serotec); human C3c: monoclonal antibody (mouse IgG_1, Chemicon), polyclonal antibody (sheep antisera, Binding Site); human C5: sheep antisera to C5 (Binding Site); human C9: sheep antisera (Binding Site); C5b-9 neoantigen: monoclonal antibody (polyC9-MA; mouse IgG_1, a gift of Dr. A.F. Michael, University of Minnesota); S-protein: monoclonal antibody (mouse IgG_1, Chemicon), polyclonal antibody (rabbit IgG, Chemicon); vitronectin receptor: monoclonal antibody to $\alpha v \beta_3$ integrin (Telios). The following antibodies were also used: fluorescein-isothiocyanate (FITC) or tetramethyl-rhodamine (TRIC)-labelled affinity-purified goat anti-mouse IgG (Cappel), FITC-labelled affinity-purified goat anti-rabbit IgG, or rabbit anti-sheep IgG (Cappel), FITC-labelled goat anti-human IgG, IgA, and IgM (Cappel), and colloidal gold-conjugated rabbit anti-sheep IgG (EY Laboratories), and goat anti-mouse IgG and goat anti-rabbit IgG (Janssen).

The reactivity of antibodies was checked by enzyme-linked immunoabsorbent assay, Western blotting, and absorption tests. Purified antigens, S-protein (Iwaki Glass), type IV collagen, laminin and fibronectin (Bethesda Research Laboratories), and human whole C3 (Diamedix) were separately coated on microtitre-plate wells at concentrations of 0.001–10 μg/ml. The polyclonal antibodies were then reacted with peroxidase-labelled rabbit anti-sheep IgG and goat anti-rabbit IgG (Cappel). The monoclonal antibodies were reacted in the biotin-avidin system as previously reported (Yoshioka et al. 1989). The monoclonal and polyclonal anti-S-protein antibodies reacted only with S-protein, and not with other matrix components, such as type IV collagen, laminin or fibronectin. The monoclonal and polyclonal anti-C3d antibodies did not react with human whole C3.

Human whole C3 was digested with trypsin according to the method described by Leivo and Engvall (1986). C3b was prepared by digestion at 37° C for 5 min, and C3c and C3d were prepared at 37° C for 30 min. The reaction was stopped by adding soybean trypsin inhibitor. The products were dialysed and lyophilized. SDS-polyacrylamide gel electrophoresis, followed by Western blotting, was performed as described previously (Yoshioka et al. 1989, 1990). Proteins transferred onto a nitrocellulose membrane were reacted with anti-C3d monoclonal or polyclonal antibodies or anti-C3c polyclonal antibodies. After being rinsed, they were reacted with peroxidase-labelled goat anti-mouse IgG or rabbit anti-sheep IgG for 1 h at room temperature. Anti-C3d monoclonal and polyclonal antibodies reacted with C3b and C3d, and anti-C3c polyclonal antibodies reacted intensely with C3 and its degradation products.

The polyclonal and monoclonal antibodies to S-protein were incubated with purified S-protein for 1 h at room temperature, and then overnight at 4° C, and examined by immunofluorescence. The binding of the antibodies to the renal tissues was completely inhibited. The polyclonal antibodies to C3c, C3d and S-protein were incubated with the renal tissues overnight at 4° C, and then

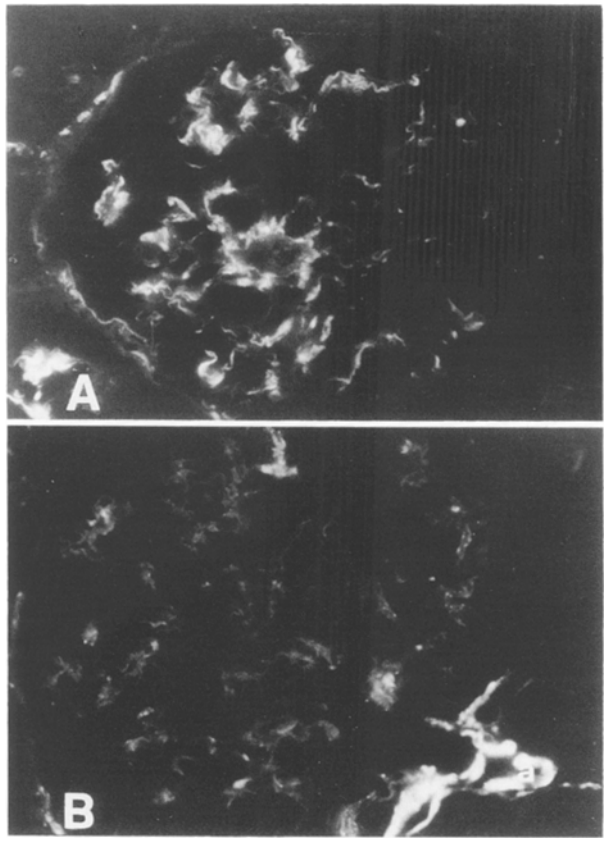

Fig. 1 A, B. Distribution of C3d and S-protein in normal human kidneys. **A** Immunofluorescent staining with a monoclonal antibody to C3d. C3d is positive along the glomerular basement membrane and part of the tubular basement membrane. **B** Immunofluorescent staining with a monoclonal antibody to S-protein. S-protein is weakly positive along the glomerular capillaries and a positive granular pattern is present in the mesangium. Arteriole (*a*) is intensely stained. Original magnification. × 400

examined by immunofluorescence, using their own monoclonal antibodies. The result was negative.

Direct and indirect immunofluorescent staining was performed as previously described (Yoshioka et al. 1989, 1990): the biopsied tissues were quickly frozen in acetone-dry ice or isopentane-liquid nitrogen and embedded in OCT compound. They were cut into 4-μm-thick sections, fixed with cold acetone at 4° C, washed with PBS, and reacted with the primary antibody, followed by incubation with the appropriate secondary antibodies. Dual label staining was performed, using FITC-labelled and TRIC-labelled secondary antibodies, as reported previously (Yoshioka et al. 1990).

Immunoelectron microscopy was performed as reported previously (Yoshioka et al. 1989, 1990). Colloidal gold-conjugated antibodies were used as the secondary antibody.

Results

In normal kidney C3d was stained in a linear pattern along the GBM, and segmentally along the Bowman's capsule and the TBM (Fig. 1A). C3d was also intensely positive in the small blood vessels. C3c was negative in the glomeruli, but weakly positive in the small blood vessels. C5b-9 neoantigen was found in a granular pattern in the small blood vessels and in some parts of the TMB, along with weak mesangial staining. S-protein was positive in a linear pattern along the GMB, in the mesangium, and intensely so in the vascular pole of the glomeruli and small blood vessels (Fig. 1B). Parts of Bowman's capsule were also positive. Immunofluorescence for vitronectin receptor was positive in the arterioles, parts of Bowman's capsules, and some tubular epithelial cells, and weak in the glomerular measangium.

The localization of C3d and S-protein was further examined by immunoelectron microscopy, using isolated basement membranes (Fig. 2): C3d was observed unilaterally only on the epithelial side of the GBM. Both sides of the isolated GBM were stained for S-protein, exhibiting intense labelling on the epithelial side and weak labelling on the endothelial side.

In the tissues from patients with minimal change nephrotic syndrome, the staining pattern of C3d, C3c, C5b-9 neoantigen and S-protein was almost identical to that in normal tissues. In other types of glomerulonephritis, the staining tended to show an increase in both intensity and distribution. C3d was deposited more diffusely in the glomerulus, compared to C3c deposits (Fig. 3). Staining for C3d, C3c, C5b-9 neoantigen, and S-protein was concordant in the mesangial immune deposits in IgA nephritis and Henoch-Schonlein purpura nephritis. In the tissues of mesangial proliferative (non-IgA) glomerulonephritis, haemolytic uraemic syndrome and oligomeganephronia, S-protein was predominantly deposited in the mesangium. In the tissues of membranous nephropathy, S-protein and complement components were positively stained in a fine glomerular pattern along the glomerular capillary walls (Fig. 4A). In the tissues of membranoproliferative glomerulonephritis and lupus nephritis, intense deposition of the complement components and S-protein was observed in the mesangium and along the glomerular capillaries (Fig. 4B). The sclerotic portion of the tissues in focal glomerulosclerosis and reflux nephropathy were intensely stained for C3d, C3c, C5b-9 neoantigen and S-protein.

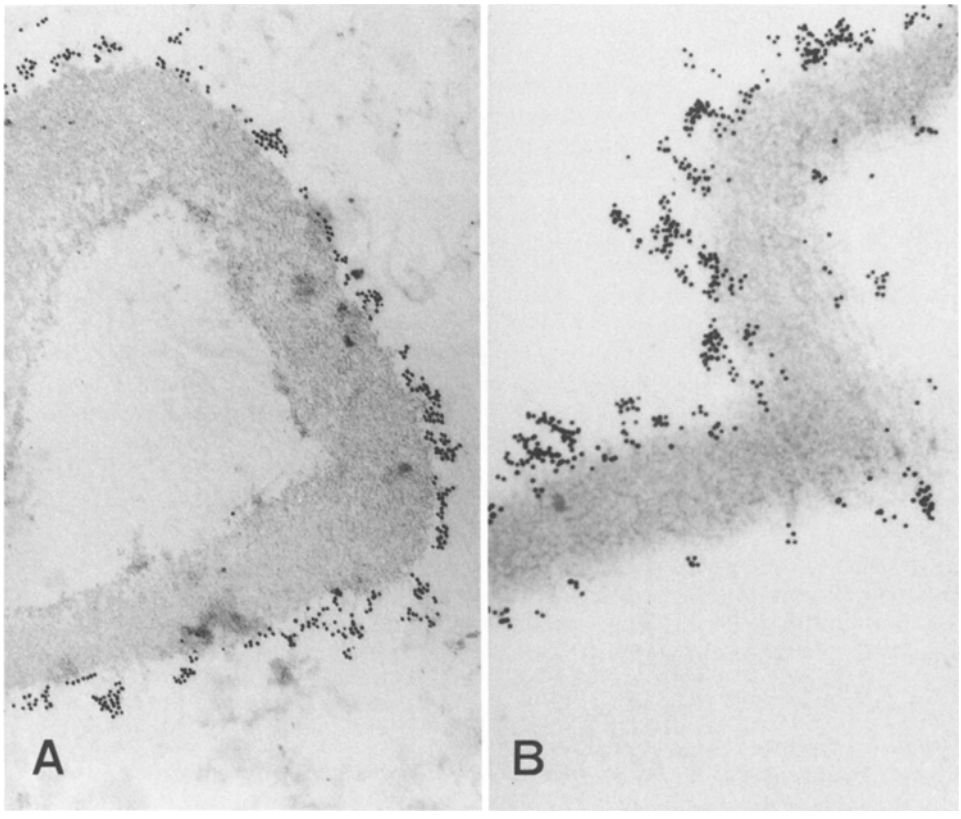

Fig. 2A, B. Immunoelectron microscopic observation of the isolated glomerular basement membrane, stained for C3d and S-protein, using a colloidal gold technique. **A** C3d is present on the epithelial side (denser and smoother than the other side) of the glomerular basement membrane, but is virtually absent from the endothelial side (gold particle size: 15 nm). **B** S-protein is localized on both the epithelial and endothelial sides of the glomerular basement membrane (gold particle size: 10 nm). Original magnification, **A**: 12000, **B**: ×10000

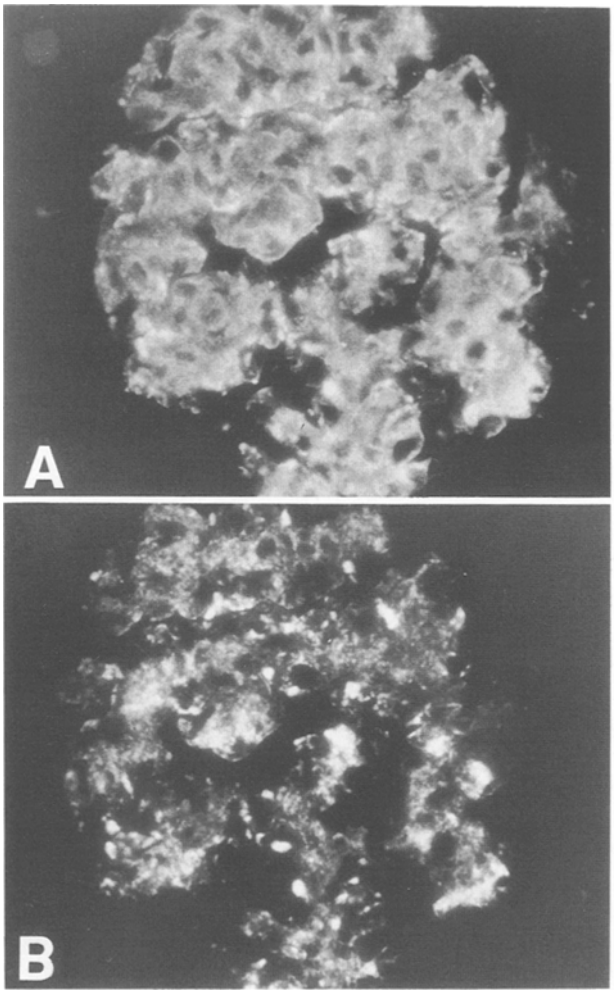

Fig. 3A,B. Dual immunofluorescent staining for C3d and C3c. C3d (**A**) and C3c (**B**) deposition in a kidney section from a patient with IgA nephritis, showing mesangial distribution of C3d and C3c, and glomerular capillary staining for C3d. Original magnification. ×400

The staining for S-protein tended to become more intense as the mesangial matrix expanded, and was accompanied by intense staining for C5b-9 neoantigen (Fig. 4C, D). Immunofluorescence for vitronectin receptor was positive, particularly in the expanded mesangium of mesangial proliferative types of glomerulonephritis, and was also positive within the crescents. Dual immunofluorescent staining showed that mesangial localization of vitronectin receptor was occasionally concordant with that of S-protein in the glomeruli (Fig. 4E, F).

In the TBM, C3c deposits were rare. C3d and C5b-9 neoantigen were deposited more intensely in a granular pattern in the tissues of renal diseases than in the tissues of the normal controls. S-protein showed intense and diffuse labelling of the TBM in a linear pattern.

Immunoelectron microscopic observation revealed that S-protein was positive in the GBM and mesangium, and being especially intense in the electron dense deposit found in the subepithelial, subendothelial, or mesangial regions (Fig. 5).

The correlation between C3d or S-protein deposition and histological or clinical findings was examined in mesangial proliferative types of glomerulonephritis, including IgA nephritis, Henoch-Schonlein purpura nephritis, and mesangial proliferative (non-IgA) nephritis. The amount of deposition of S-protein in the mesangium correlated positively ($p < 0.05$) with mesangial matrix increase. There was no significant correlation between S-protein deposition and the severity of haematuria and proteinuria, the presence of hypertension, or creatinine clearance.

Discussion

In the present study, S-protein was localized immunohistochemically in association with the activated complement components, C3d and C5b-9, and also with its receptor, vitronectin receptor. Both the presence and the absence of S-protein in the normal glomerulus have been reported (Falk et al. 1987; Tomino et al. 1987; Bariety et al. 1989). Our findings support the presence of S-protein in the normal human glomerulus. S-protein normally exists in the circulating blood and no evidence has been produced for its synthesis by the glomerular cells themselves. Thus, it seems that glomerular S-protein is derived from plasma, through its affinity to the glomerular components, in a manner analogous to the glomerular deposition of amyloid P and fibronectin.

The staining of S-protein was more pronounced in the tissues associated with renal diseases, particularly in tissues with marked mesangial expansion and mesangial or subepithelial immune deposits. S-protein was generally co-localized with C3d and C5b-9 neoantigen within the immune deposits, suggesting local activation of the terminal complement pathway, followed by in situ S-protein binding. Studies by Bhakdi et al. (1988) suggest that S-protein can interact with membrane-bound C5b-9, exhibiting a weak function as MAC. Another explanation of the findings of co-localization is that C5b-9 and S-protein is not a real complex, and that the co-existence is only apparent. This possibility is supported by our recent findings in tissues from patients with congenital C9 deficiency and IgA nephritis, where S-protein was clearly deposited in the mesangial immune deposits without accompanying C5b-9 deposition (Yoshioka et al. 1992). S-protein deposits in tissues with C5b-9 could be derived from circulating SC5-9. If so, the SC5b-9 deposited may be inert. However, trapping of circulating SC5b-9 complex into the glomerulus is unlikely, particularly in the case of SC5b-9 present in subepithelial immune deposits, since its molecular mass is huge (approximately 1×10^3 kDa), far too large to be filtered across the glomerular basement membrane barrier. Vitronectin receptor expression was evident in the expanded mesangium of glomerulonephritis. It is possible that S-protein might be derived, at least in part, from interaction with an integrin receptor.

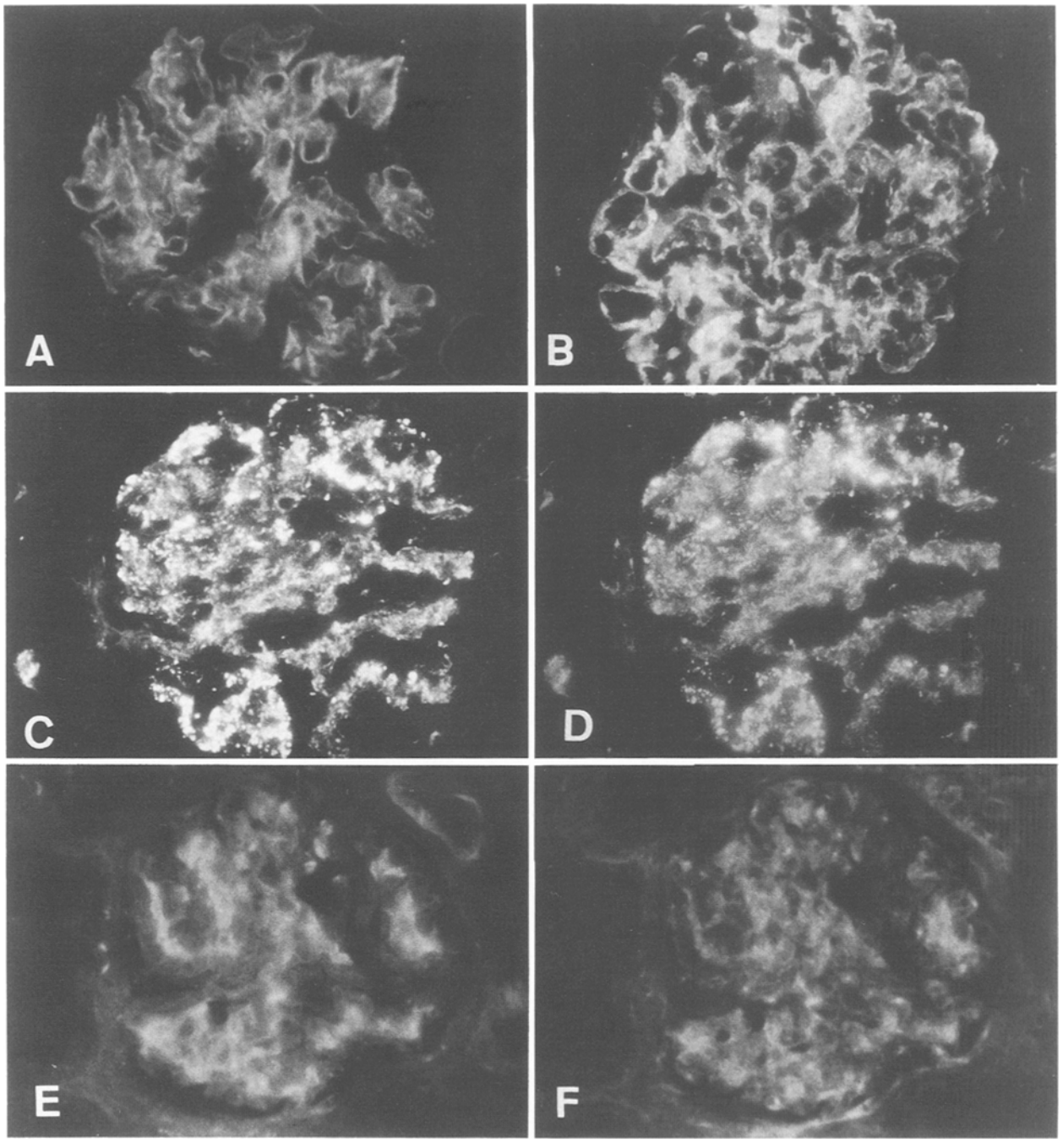

Fig. 4A–F. Immunofluorescent observations of S-protein in association with C5b-9 and vitronectin receptor. **A** S-protein staining in membranous nephropathy. Note S-protein deposits along the glomerular capillary walls. **B** S-protein staining in lupus nephritis (WHO Class IV). Note diffuse distribution of S-protein in the glomerulus. **C, D** Dual staining for S-protein (**C**) and C5b-9 neoantigen (**D**) in kidney tissue from a patient with IgA nephritis. Note co-localization of S-protein and C5b-9 neoantigen in the expanded mesangium. **E, F** Dual staining for S-protein (**E**) and vitronectin receptor (**F**) in kidney tissue section from a patient with IgA nephritis. Note the co-localization in the glomerulus. Original magnification $\times 400$

The present study demonstrated the deposition of C3d in normal and nephritic tissues. C3d was present in the GBM, TBM, vascular walls, and a part of Bowman's capsule, and its distribution was different from that of C3c. This observation was in agreement with those in studies by Leivo and Engvall (1986), who showed C3d deposition in GBM and placental basement membrane in the absence of signs of local complement activation or immune complex deposition. They suggested interaction between C3d and the components of the GBM, especially laminin. Our immunoelectron microscopic observations, using isolated GBM, revealed

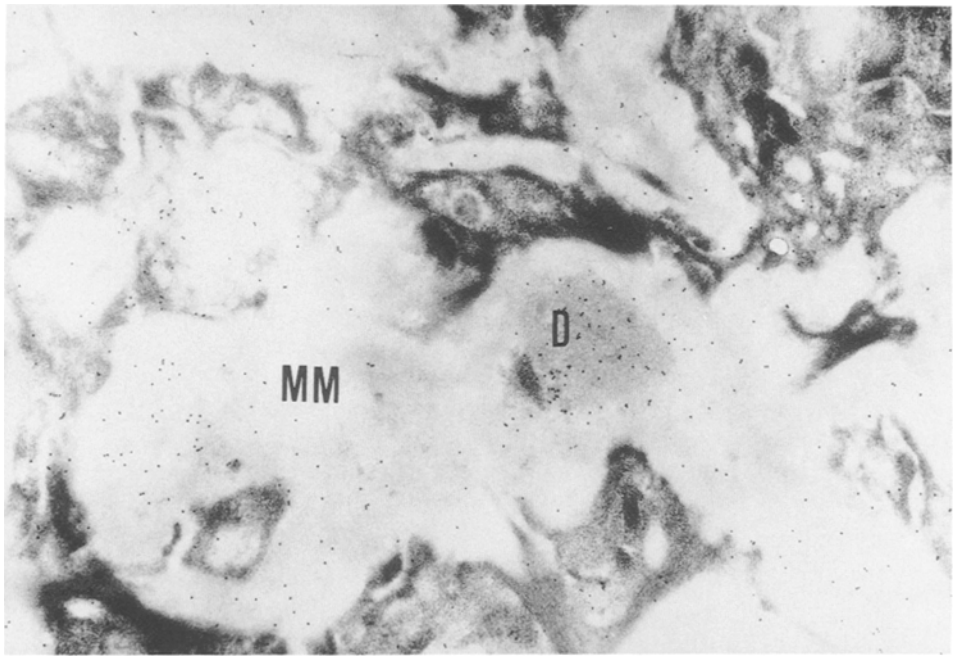

Fig. 5. Immunoelectron micrograph of a part of a glomerulus from a patient with lupus nephritis; the specimen is stained with a monoclonal antibody to S-protein and colloidal gold-conjugated secondary antibody. Note intense labelling within a mesangial electron-dense deposit (*D*), and weak labelling in the mesangial matrix (*MM*). Original magnification, ×12000

that C3d was localized exclusively on the epithelial side of the GBM. The reason for this is not clear, but the high affinity of C3d to a certain basement membrane component organizing the epithelial side of the GBM, or the stagnation of C3d on the epithelial side could explain it.

Vitronectin is a component of the extracellular matrix, to which cells adhere via mediation with a ligand, namely integrin receptors. It has recently been revealed that interaction between the extracellular matrix and cells can modify the cells' function and behavior (Madri and Basson 1992). In addition to its regulatory role in the terminal complement pathway, S-protein or vitronectin might play a role in glomerular injury or in its repair, through this mechanism. The need for future study to further elucidate the role of S-protein in human glomerulonephritis is apparent.

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