Localization of S Protein and Its Relationship to the Membrane Attack Complex of Complement in Renal Tissue

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The S protein (S) binds to the attack complex of complement (C5b-9) in plasma preventing cytolysis. Using immunofluorescence microscopy, the authors determined the distribution of S in human renal tissue and its relationship to C5b-9, immunoglobulins, C3, albumin, and fibronectin. They examined normal and diseased human kidney tissue from patients with several forms of glomerulonephritis, diabetic nephropathy, and arterionephrosclerosis. S and C5b-9 were found in all diseased tissues; their amounts and distribution directly correlated with severity and location of injury. S and C5b-9 were colocalized in all immune deposits and in all injured glomeruli, tubular basement membranes, and vessel walls. Other than within immune deposits, S and C5b-9 were usually not colocalized with C3. This study demonstrates that S is deposited in areas of tissue injury and thus may participate in the pathogenesis of renal damage. Because in tissue S and C5b-9 are always associated, the attack complex in tissue must either be derived from the circulation as SC5b-9 or it must be capable of binding S after the formation *in situ* of C5b-9. (Am J Pathol 1987, 127:182–190)

THE CYTOLYTIC MEMBRANE attack complex, C5b-9(m), develops during complement activation on lipid bilayers. This amphiphilic macromolecule inserts into lipid membranes via lipophilic binding sites. The S-protein (S) is an acid glycoprotein found in human plasma.[1] Binding of S to the attack complex of complement during its assembly in plasma results in the formation of a cytolytically inactive complex, SC5b-9.[2] *In vitro,* S covers exposed apolar surfaces on fully assembled C5b-9(m), thereby converting an amphiphilic structure into a hydrophilic macromolecule.[3] Whether this *in vitro* effect of S on C5b-9(m) occurs *in vivo* is not known.

C5b-9 has been immunohistochemically located in renal tissues from patients with immune- and nonimmune-mediated renal disease.[4,5] In selected experimental models of immune mediated glomerulonephritis, the attack complex plays an important pathogenic role.[6,7] Detection of the complex in tissue depends on antibodies that recognize neoantigens present in the attack complex but not present on native complement components. All of these reagents recognize C5b-9 incorporated in SC5b-9 as well as C5b-9(m).[8] Thus the immunohistochemically identified complex may be present as a result of local complement activation or deposition from the circulation. The possibility that SC5b-9 is deposited from the circulation must be considered because it has been detected in the plasma of patients with active glomerulonephritis.[9,10]

We sought to determine the presence and location of the S protein in normal and diseased human renal tissue. Because the S protein is the only immuno-

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histochemical difference between SC5b-9 and C5b-9(m), we compared the distribution of S with that of C5b-9, C3, and IgG, IgM, and IgA. Because many anionic serum proteins bind to the glomerular capillary wall,[11] we compared the distribution of S with that of similarly anionic albumin. These studies were performed in human renal tissue having no pathologic evidence of disease and in a variety of immune and nonimmune mediated lesions.

Materials and Methods

Antibodies

The characterization of the monoclonal antibody to a neoantigen of C9 present in C5b-9(m) and SC5b-9 has been previously documented.[4] The monoclonal antibody to S which was made from purified S was a kind gift from Cytotech (San Diego, Calif). This monoclonal antibody is specific for purified S, and it is also specific for S incorporated into SC5b-9. The immunoglobulin fraction of a polyclonal goat antibody to human S was absorbed with S-deficient human serum[2] and then rhodaminated by conventional techniques. The monoclonal and polyclonal anti-S antibodies had identical staining patterns in human renal tissue. Polyclonal fluoresceinated antibodies to human C3, IgG, IgM, and IgA were obtained from Meloy (Springfield, Va), and to C5 from Atlantic Antibody (Scarborough, Me). Fluoresceinated anti-human albumin, human plasma-absorbed goat anti-mouse Ig (heavy- and light-chain-specific) and human plasma-absorbed rabbit anti-goat Ig (heavy- and light-chain-specific) were obtained from Cappel Laboratories (Malvern, Pa). A monoclonal antibody to fibronectin was also used.[12]

Tissue Sections

Renal tissue was obtained for this investigation as part of clinically indicated diagnostic studies. We examined 6 renal biopsy specimens with no morphologic lesions on light, immunofluorescence, or electron microscopy; 7 with minimal change disease; 11 with membranous glomerulopathy; 11 with IgA glomerulonephritis (GN); 4 with postinfectious GN (characteristic clinical history and typical epimembranous humps along the glomerular basement membrane [GBM]); 8 with proliferative systemic lupus nephritis; and 3 with Type I membranoproliferative GN (MPGN). In addition, 10 cases of diabetic nephropathy were examined in patients with known diabetes, clinical nephrosis, and the absence of other glomerular disease. Six cases of arterionephrosclerosis and 4 cases of transplant allograft rejection were also studied.

Immunofluorescence Studies

For immunofluorescence microscopy, blocks of tissue were snap-frozen in liquid nitrogen, embedded in OCT, and stored at −70 C until sectioned. Some specimens were held in Michell's transport medium prior to freezing. Four-micrometer sections were stained with the antibodies described above.[4,13] Preparations were examined with the use of a Lietz Orthoplan microscope equipped for incident light fluorescence microscopy. Immunofluorescence studies were performed on serial or step sections for evaluation of the relative positions of the antigens. The intensity and distribution of immunostaining for each protein were assessed on an arbitrary scale of 0–4+. Specific co-localization of the neoantigen of C9 and S was performed by sequentially incubating the tissue with anti-neoantigen of C9, fluoresceinated anti-mouse Ig, and rhodaminated polyclonal anti-S antibody.

Results

The amount of immunostaining for S protein in renal tissues was directly proportional to the degree of morphologic injury assessed by light-microscopic evaluation (Figure 1). There were negligible amounts of S in cases of no lesion and minimal change disease (MCD). In contrast, the intensity of anti-S immunoreactivity was most marked in tissues in which there were plentiful immune deposits (systemic lupus erythematosus [SLE] or MPGN) or large areas of mesangial expansion and glomerulosclerosis (diabetes mellitus).

Along glomerular capillary walls, S was not detectable in cases of no lesion and MCD (Figure 1). In diseases in which there were minimal alterations of the GBM (IgA GN, allograft rejection, and arteriosclerotic disease), S was linearly distributed in a trace to 1+ intensity along the endothelial surface of the GBM. In this instance, the pattern of albumin immunoreactivity was similar to that of S, but with a much greater intensity than S. The linear distribution of S along the GBM was most marked in cases of diabetes mellitus (Figure 2A). In membranous nephropathy, S was present in immune deposits within and along the epithelial surface of the GBM (Figure 3A and B). In SLE GN, MPGN, and postinfectious GN, S was localized in subepithelial and subendothelial immune deposits.

In the mesangium, S was only minimally present in the hilar region of no lesion and MCD cases. The

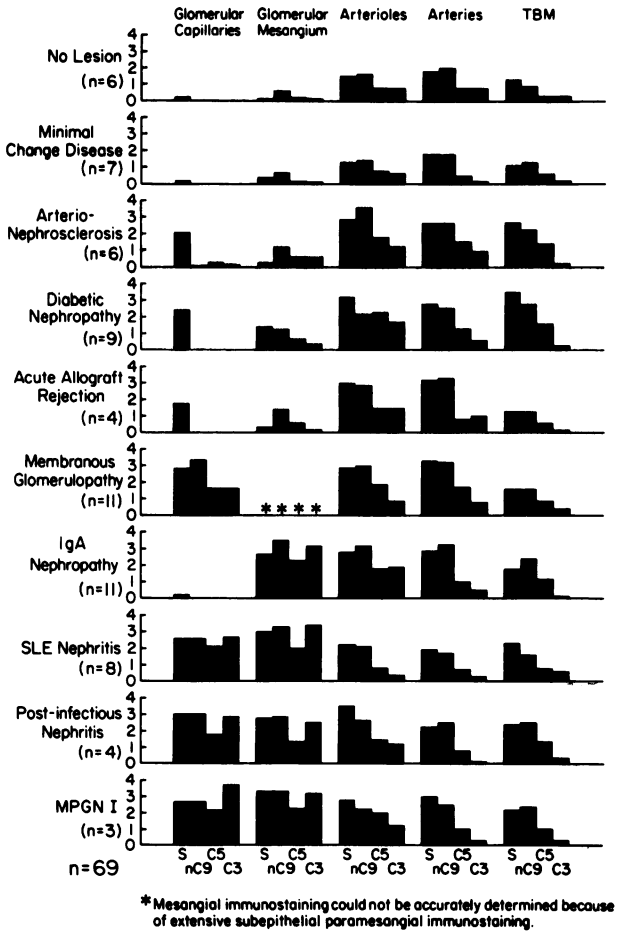


Figure 1—Comparison of S, neoantigen of C9 (NC9), C5 and C3 immunoreactivity in glomerular capillaries and mesangium, arterioles, arteries, and tubular basement membranes (TBM) in renal tissues without lesions and immune- and non-immune-mediated human renal diseases.

intensity of anti-S staining increased with advancing mesangial expansion and glomerulosclerosis. This was best exemplified in diabetic nephropathy and arterionephrosclerosis. S was present in mesangial immune deposits in immune-mediated glomerular diseases. It was intensely localized in the mesangium in IgA (Figure 4A), SLE, postinfectious and Type I MPGN.

In arterioles and arteries, S was present throughout vessel walls (Figure 5A). There was relatively less S immunoreactivity in no lesion and MCD cases when compared with the high intensity and homogeneous distribution seen in all other disease groups. Along tubular basement membranes, S was present in a focal granular pattern (Figure 6). The extent of its distribution and the intensity of anti-S staining directly correlated with the degree of tubulointerstitial injury.

The presence of C5b-9 was determined by the colocalization of C5 with the neoantigen of C9 present in the attack complex. These two antigens were always found together in all loci and in all disease groups. This observation has been previously reported, as well as the observation that all other constituents of the attack complex are present with C5 and neoantigen of C9.[4] Thus the colocalization of C5 with the neoantigen of C9 immunohistochemically identifies the C5b-9 complex.

When S was linearly distributed along the GBM, C5b-9 was not present. However, in all other loci and in all disease groups S and C5b-9 were colocalized. In immune mediated disease, S and C5b-9 were ubiquitously found together in immune deposits. The intensity of immunostaining for S, C5 and neo-C9 were similar (Figure 1). S and C5b-9 were colocalized, for example, in intra- and epimembranous immune deposits in membranous nephropathy (Figure 3); along with C3 and IgA in the mesangium of IgA GN (Figure 4B); in subepithelial and subendothelial immune deposits in SLE; in epimembranous humps in postinfectious GN; and in MPGN. Wherever S and C5b-9 were present in immune deposits, C3 was present, albeit with lesser intensity of immunoreactivity.

In both nonimmune and immune mediated diseases, S and C5b-9 were always colocalized in regions of mesangial expansion and glomerulosclerosis. In these loci, C3 was not regularly present. This was especially notable in vessel walls (Figure 5) and along tubular basement membranes where S and C5b-9 were almost invariably deposited together in the absence of C3 or immunoglobulin.

The S protein is an adhesive protein which binds to a variety of surfaces. The gene sequence for the S protein has recently been established and found to be homologous with vitronectin.[14] Vitronectin is an adhesive extracellular matrix protein similar in nature to fibronectin.[15] The distribution of vitronectin in tissues has been compared with that of fibronectin.[16] We sought to determine whether the localization of fibronectin was similar to that of S. Fibronectin was present in all cases studied in a 2–3+ intensity in the mesangium, in a trace distribution along the glomerular capillary walls, and in vessel walls usually along the luminal surface. It was not present along tubular basement membranes. Therefore, the distribution of fibronectin was entirely unlike that of S and C5b-9 in all of the diseases studied (Figure 3H).

Discussion

The distribution of the S protein and the intensity of its immunoreactivity were evaluated in normal human renal tissues and in immune and nonimmune diseases. The relative amounts of S, as assessed by

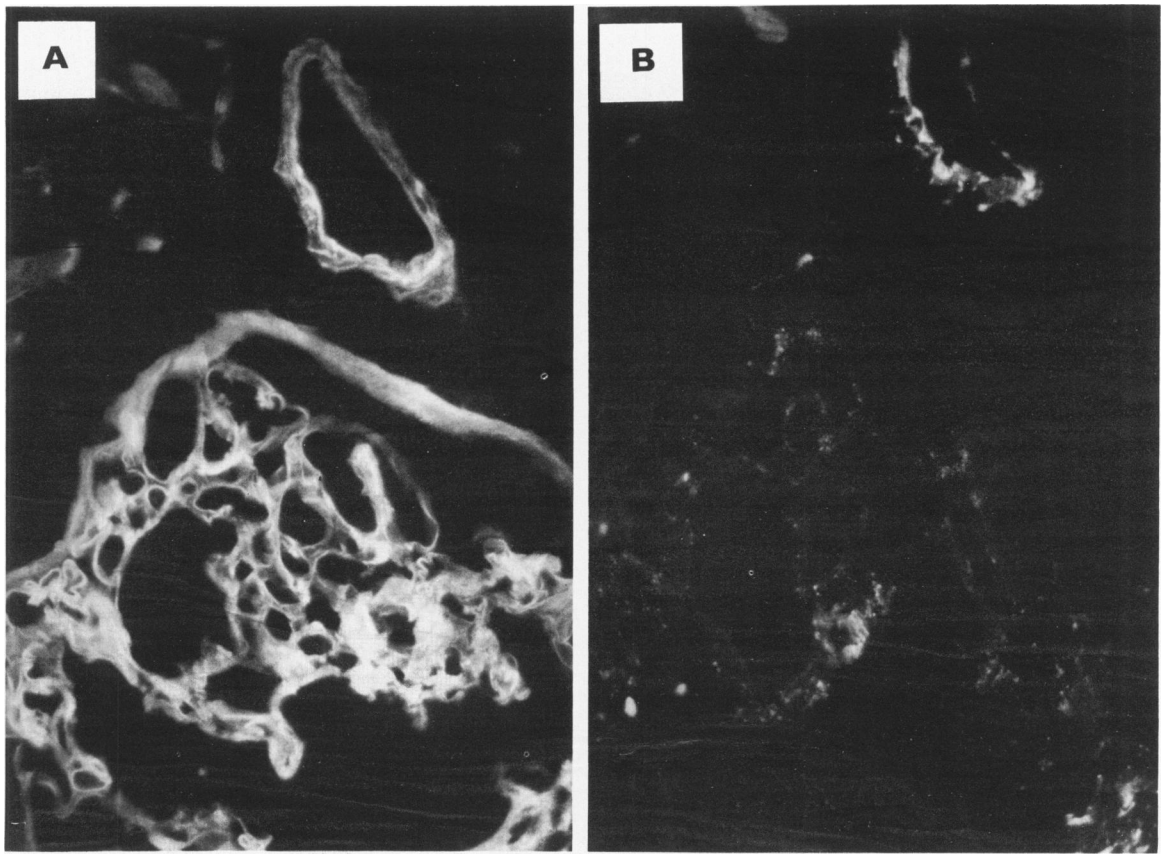


Figure 2—Step sections of a glomerulus and arteriole from a patient with diabetic glomerulosclerosis. (×360) **A**—Immunostaining for S protein showing staining of glomerular mesangium and basement membranes and the arteriole. **B**—Immunostaining for C9 neoantigen showing granular staining of glomerular masangium and the arteriole.

immunohistochemical techniques, were directly proportional to the severity of injury. The amount of S ranged from undetectable in no lesion cases to a 4+ intensity in areas of injured glomeruli, tubules, and blood vessel walls.

The presence of S in a linear pattern along the GBM, primarily in nonimmune diseases, was similar to that seen with albumin. S has isoelectric points in the range pH 4.75–5.25.[1,2] Melvin et al[11] have observed that many anionic proteins bind along the glomerular capillary wall, whereas proteins with isoelectric points greater than pH 6.0 do not. This process is accentuated in certain diseases, especially diabetic nephropathy. The linear distribution of S in this location may be attributable to an electrical charge interaction. In these loci, S was not associated with C5b-9.

In all immune-mediated renal lesions, S and C5b-9 were localized together with similar intensity in immune deposits. Within immune deposits, S and C5b-9 were present with C3 and immunoglobulin. Presumably in these areas the attack complex is locally generated by classical or alternative pathway activation. S and C5b-9 were also colocalized in diseased mesangium, injured tubular basement membranes, and vessel walls in all diseases (immune- and non-immune-mediated). In these loci, in contrast to our findings within immune deposits, S and C5b-9 were usually not colocalized with C3. This was especially true along tubular basement membranes and in vessel walls.

The invariable colocalization of S with C5b-9 indicates that C5b-9(m) does not exist in tissues without association of S. This contention is based on the specificity of the antibodies employed in this study. The antibody to a neoantigen of C9 binds to activated C9 and not to native complement components, nor does it bind to any intermediate complex in attack complex formation.[4] The anti-S monoclonal binds to S and S incorporated into SC5b-9. It does not bind to complement components C5 through C9.

The complex of S in tissue with C5b-9 could be derived from two sources. Either SC5b-9 formed in the blood compartment deposits in tissues, or C5b-9(m) is locally formed by complement activation

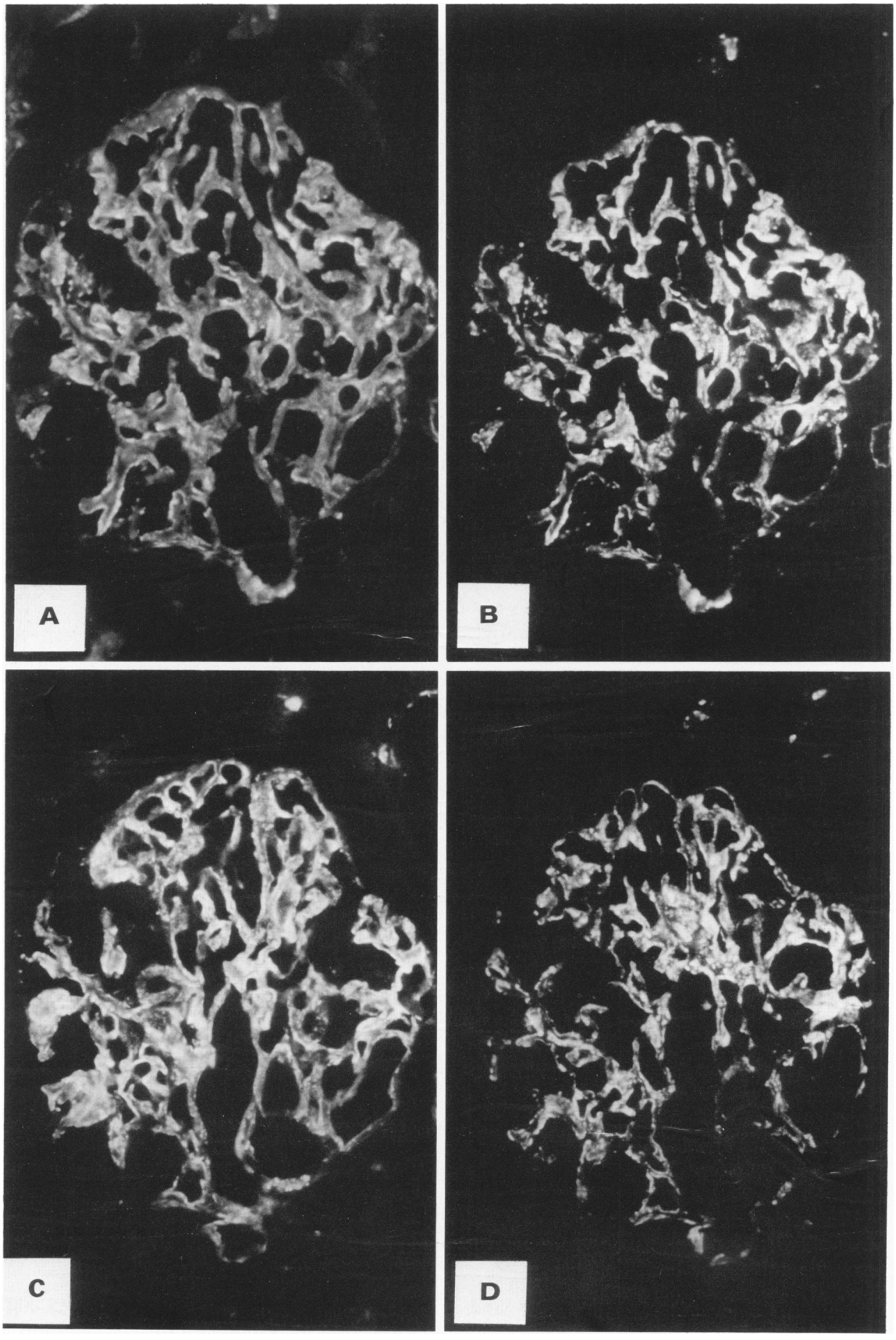
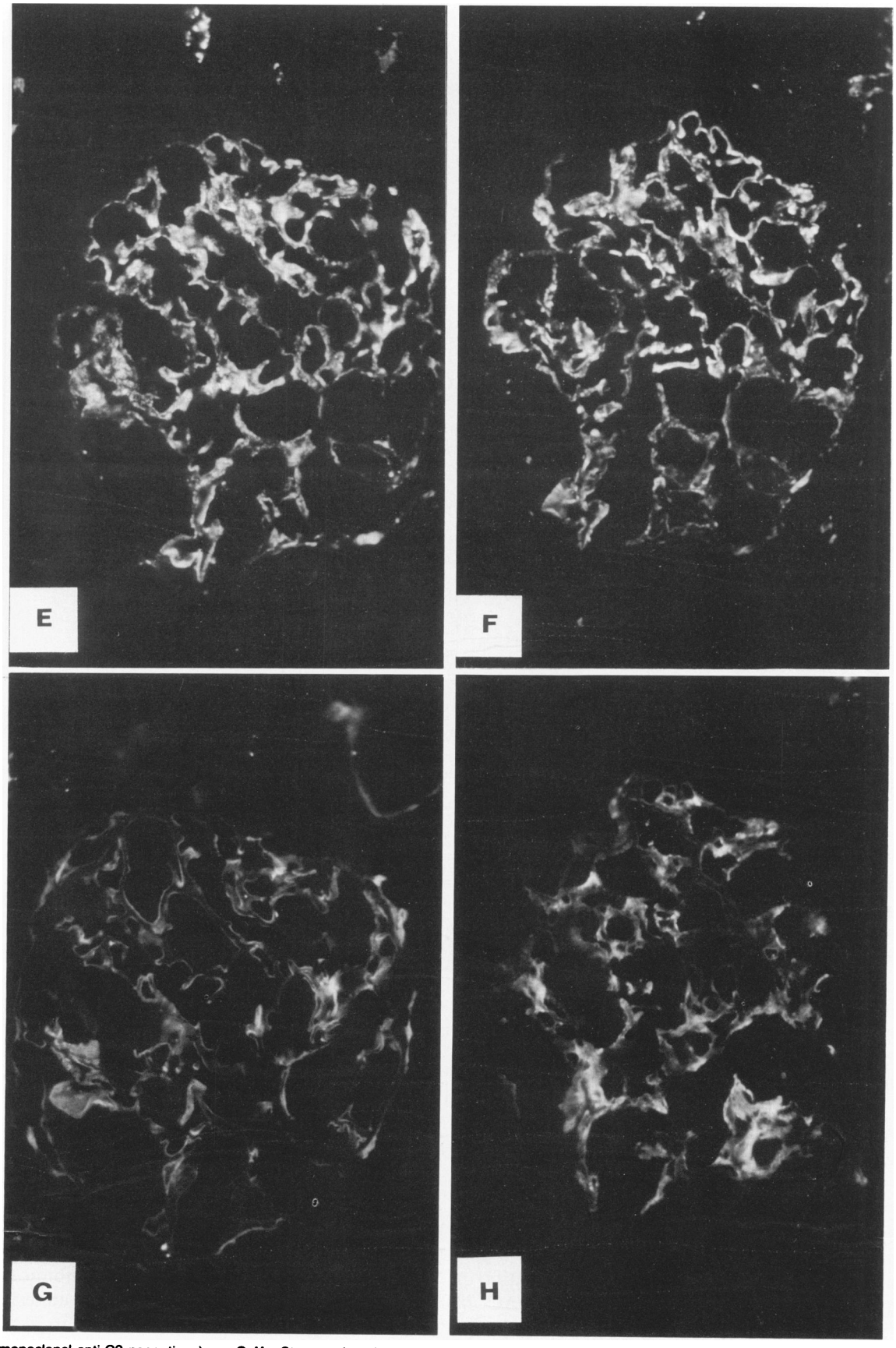


Figure 3—Immunofluorescence microscopy of step sections of a glomerulus from a patient with idiopathic membranous glomerulopathy. (×340) **A** and **B**—Same section dual stained respectively for S protein (rhodaminated polyclonal goat anti-S protein) and C9 neoantigen (fluoresceinated second antibody to



mouse monoclonal anti-C9 neoantigen). **C–H**—Step sections immunostained with monoclonal anti-S protein (**C**), monoclonal anti-C9 neoantigen (**D**), polyclonal anti-C3 (**E**), polyclonal anti-C5 (**F**), polyclonal anti-albumin (**G**), and monoclonal anti-fibronectin (**H**).

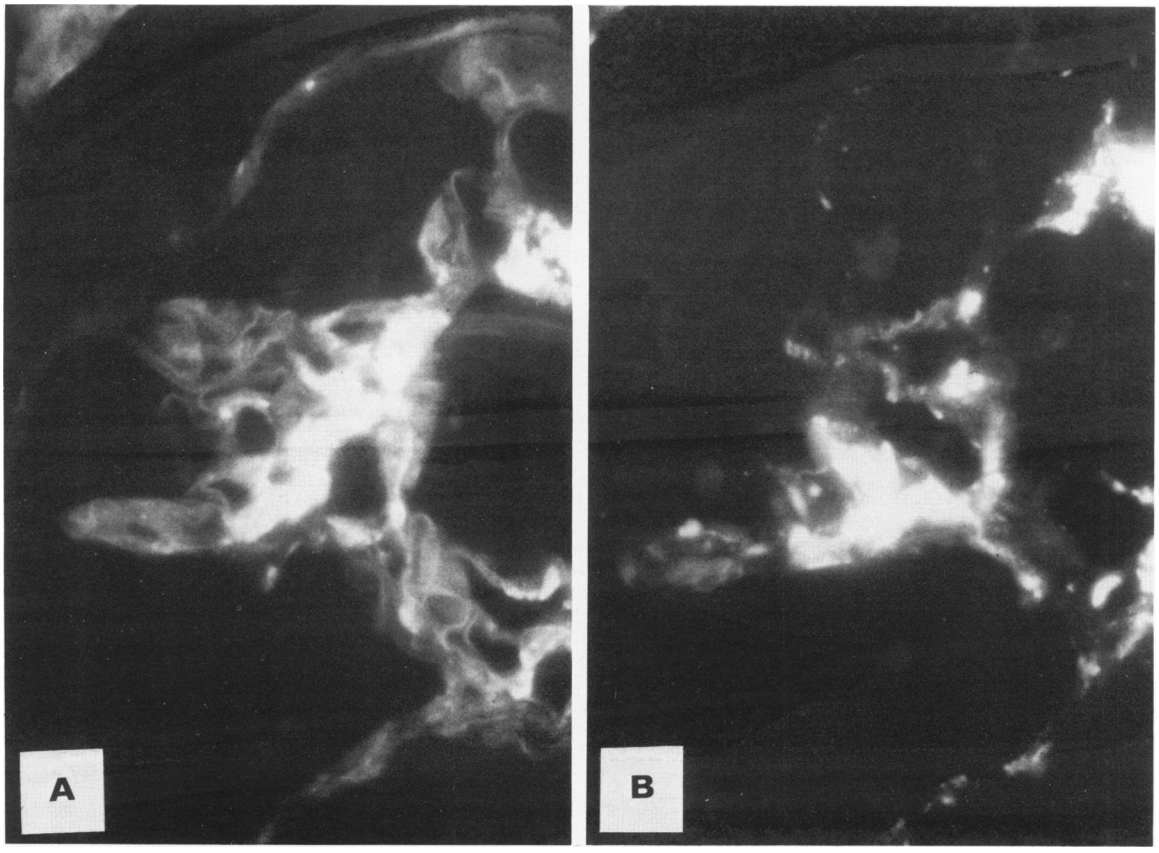


Figure 4—Step sections of a glomerular segment from a patient with IgA nephropathy immunostained for S protein (**A**) showing intense mesangial immune deposit staining and low-intensity basement membrane staining and immunostained for C9 neoantigen (**B**), showing intense staining of immune deposits. (×1000)

and then complexed to S. Both processes may occur. The presence of SC5b-9 in the plasma of patients with lupus nephritis[9] and other immune-mediated diseases[10] raises the possibility of its deposition from the circulation into tissue. Moreover, the observation of S and C5b-9 localization without association of C3 in vessel walls suggests that the circulating complex may be deposited, rather than generated by local complement activation. However, S must also be complexing with the membrane attack complex after local complement activation. The strongest support for this contention is derived from our observation of S and C5b-9 colocalization in intra- and epimembranous deposits in 11 cases of membranous glomerulopathy. This disease process is putatively caused by *"in situ"* immune complex formation.[17] Because C5b-9 is presumably generated by classical pathway activation in the immune deposit, S must be bound to the MAC *"in situ"* as well. Moreover, the large size of SC5b-9 (approximately 1 million daltons) renders unlikely the possibility of the filtration of the intact molecule across the glomerular capillary wall.

The *in vivo* observation of S binding to preformed C5b-9 has an *in vitro* experimental basis.[3] When S binds to isolated and purified C5b-9(m), the complex changes from an amphiphilic to a hydrophilic structure by the covering of exposed apolar surfaces. This complex has been given the name SC5b-9(m). As a corollary, the hydrophilic-amphiphilic transition also may occur when S is proteolytically cleaved from C5b-9, which thereby generates a cytolytically active complex.[18,19] This immunohistochemical study would support these *in vitro* observations, suggesting that S binds to preformed C5b-9(m) *in vivo.*

The gene sequences for S and vitronectin have a high degree of homology.[14] Vitronectin is an extracellular matrix molecule to which cells adhere. Fibronectin, laminin, and collagens may have similar capabilities.[15] We were concerned that the distribution of S in the kidney has been similar to other adhesive proteins. Comparative studies of S with fibronectin and the known distribution of laminin and collagen types I through V in normal and diseased human renal tissues[12,20] exclude this concern.

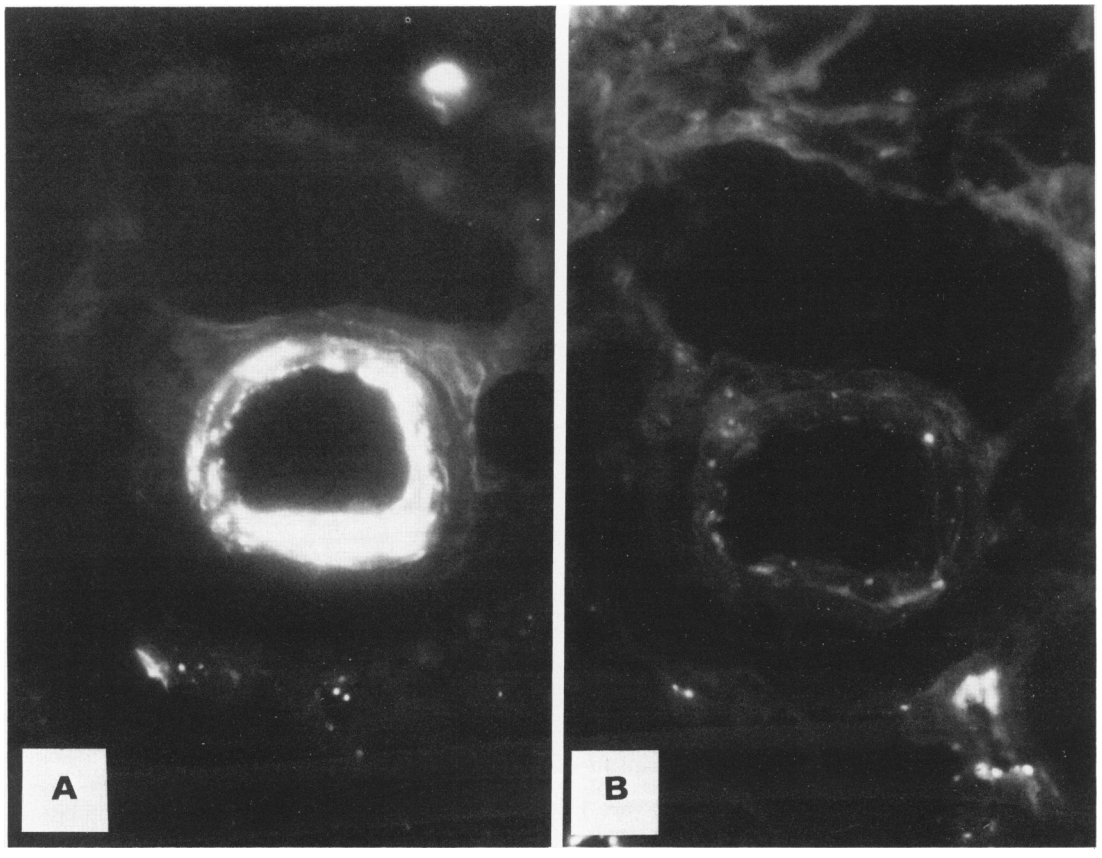


Figure 5–Step sections of an arteriole from a patient with arterionephrosclerosis showing immunostaining for S protein (**A**) and C3 (**B**). (×600)

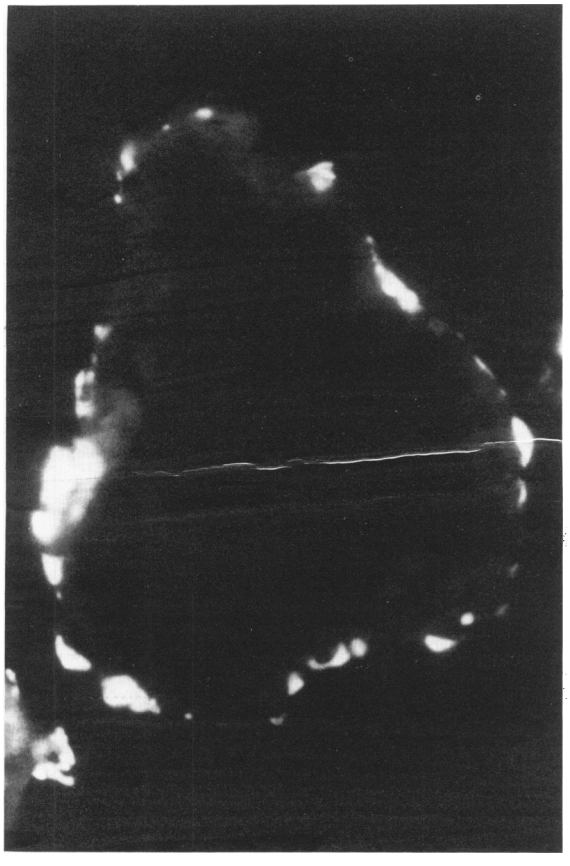


Figure 6—Immunostaining with anti-S protein of a proximal tubule from a patient with arterionephrosclerosis. (×700)

Whether S without association of C5b-9 plays a separate role in renal damage is not discernible from these studies. However, S may participate in the modulation of the damaging effects of C5b-9(m) by increasing the solubility of this macromolecule. Moreover, SC5b-9 may be deposited in tissue from the blood. Whether this fluid-phase form of the attack complex has a pathogenic effect once deposited in tissue is the subject of ongoing investigation.

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