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Immunohistochemical study of the C5b-9 complex of complement in human kidneys

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Immunohistochemical study of the C5b-9 complex of complement in human kidneys. The presence and localization of the C5b-9 neoantigens of the terminal complement sequence, of antigens expressed by cleavage fragments of C3, and of Factor H antigens have been studied by immunohistochemical techniques in morphologically normal adult human kidneys and in biopsy specimens from patients with a wide range of renal diseases with and without immune deposits. In morphologically normal kidneys, C5b-9 neoantigens were observed within all connective matrices (arteriolar media, glomerular basement membrane (GBM), mesangial matrix and tubular basement membrane). The C3d and C3g antigens of the C3dg, and C3bi cleavage fragments of C3 and Factor H antigens were found in similar locations. None of the matrices stained for immunoglobulins. Immunoelectron microscopy demonstrated that C3d, C3g, H antigens and the C5b-9 neoantigens were localized on membranous and vesicular structures embedded in the connective matrices. These structures represent cell membranes shed from adjacent cells as evidenced by their ultrastructural appearance and by the fact that those which were in close vicinity to pedicels within the GBM expressed the C3b receptor antigen, a specific marker for podocyte membranes. Formation of C5b-9 complexes in the shielded environment of connective matrices may explain their persistance over long periods of time in the absence of apparent immunopathological consequences. Biopsies from pathological kidneys were classified into three groups based on the pattern of glomerular staining with anti-C5b-9 antibodies. In the first group, a sparse mesangial labeling was seen, similar to that observed in normal kidneys. In the second group, abundant clusters of C5b-9 were seen in the same location as immune deposits. Activation of the complement system to completion could be documented in the absence of detectable C3 (C3c) antigen in glomeruli. Immunoelectron microscopy demonstrated that C5b-9 neoantigens were present on cell remnants in connective matrices in all specimens that were studied. Labeled cell remnants were present in large amounts in sclerotic matrices. C5b-9 neoantigens were constantly found on old and large immune deposits, and absent or occasionally present on recent and small immune deposits. In membranous nephropathy stage I, proteinuria appeared to be independent of the presence or absence of detectable C5b-9 neoantigens on immune deposits. Thus, the presence of C5b-9 neoantigens in pathological renal tissue does not have an univocal significance, and requires analysis of the localization of the antigens and appropriate controls in order to assess the potential role of C5b-9 in tissue damage.

Activation of the complement system results in cleavage of C3 by C3 convertases, generation of C3b, cleavage of C5 by C3b-dependent C5 convertases, and formation either of a membrane–bound C5b-9 (m) complex or of a cytolytically inactive, hydrophilic SC5b-9 complex in the fluid phase [1]. As the complement reaction progresses, C3b may be degraded into C3bi by Factor I in the presence of Factor H [2]. In the presence of C3b receptor, C3bi may in turn be cleaved by I into C3c, which is released in the fluid phase, and C3dg that remains bound to the target membrane [3–5]. The C5b-9 complex expresses neoantigens that allow its immunohistochemical detection in tissues [1, 6]. C5b-9 neoantigens and neoantigens expressed by polymerized C9 in C5b-9 complexes have been found in renal biopsy specimens from patients with systemic lupus erythematosus (SLE) [7] and in other human or experimental immune complex diseases [8, 9]. The possibility has been raised that the terminal complement complex may be directly involved in mediating tissue damage [10] and in causing proteinuria [11]. Assessment of the role of C5b-9 in renal disease is, however, complicated by the finding of C5b-9 neoantigens in the vasculature and in glomeruli in normal adult human kidney [8, 12], and in biopsies from patients with nephropathies without immune deposits [8].

In the present study, using immunofluorescence and immunoperoxidase techniques, we demonstrate that C5b-9 neoantigens, antigens expressed by the C3bi and C3dg fragments, and Factor H antigen are present in connective matrices in the morphologically–normal human kidney. At an ultrastructural level, the antigens were localized on round extracellular particles (REP) and striated membranous structures (SMS) which represent cell remnants embedded within connective matrices. C5b-9 complexes form naturally in these locations in the absence of recognizable inflammatory processes. Sixty–seven biopsy specimens from patients with various types of nephritis were also investigated for the presence and localization of C5b-9 neoantigens, C3, C3d and H antigens. In all specimens, C5b-9 neoantigens were found on cell remnants in glomerular, vascular and tubular connective matrices independently of the presence or absence of immune deposits. Staining of immune deposits with anti-C5b-9 was related to the type and the stage of glomerular lesions.

Methods

Renal specimens

Morphologically normal, adult renal tissue was obtained from six renal biopsies performed for isolated microhematuria, and

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from normal parts of two nephrectomies performed for localized renal carcinoma. Kidney specimens obtained between 1979 and 1984 from 67 adult patients (22 to 67 years old) with the clinical and immunomorphological diagnoses listed below were selected from the files of the Broussais hospital, Paris: isolated proteinuria with microhematuria (one patient), minimal change nephrotic syndrome (MCNS) (five), idiopathic membranous glomerulonephritis (IMGN) (six), drug–induced membranous glomerulonephritis (DMGN) (nine), idiopathic mesangial IgA nephropathy (seven), idiopathic IgA nephropathy associated with diabetic glomerulosclerosis (one), Schönlein–Henoch nephropathy (two), membranoproliferative glomerulonephritis (MPGN) type I (nine), diffuse crescentic glomerulonephritis (DCGN) without anti-GBM antibodies (five), SLE (nine), myeloma (two), light chain disease (LCD) (two), amyloidosis (two), scleroderma with thrombotic microangiopathy (one), Alport's syndrome (one), diabetic glomerulosclerosis (two), drug–induced acute interstitial nephritis (one), acute transplant rejection (two).

One portion of each specimen was fixed in alcoholic Bouin's fixative for light microscopy; sections were stained with Masson's trichrome or with silver solution following oxidation according to Marinozzi. Another portion of each specimen was processed for immunofluorescence as described below. Immunoelectron microscopy was performed on two normal renal specimens and on seven biopsy specimens obtained from patients with the following diagnoses: SLE nephropathy with diffuse endocapillary proliferation (type IV) (one), MPGN type I (two), Schönlein–Henoch nephropathy (one); IgA nephropathy associated with diabetic glomerulosclerosis (one), DMGN (two).

Antibodies

Affinity–purified rabbit antibodies to human C5b-9 neoantigens were prepared as described [13, 14]. Whole rabbit immune sera were first absorbed with large amounts of human serum, and specific anti-neoantigen antibodies were then isolated by a membrane absorption–desorption procedure [14]. The specificity of the antibodies for C5b-9 neoantigens was confirmed by immunoradiometric binding assays. Specific immunoglobulins accounted for approximately 65% of total protein in the preparations. Fluoresceinated (FITC) goat IgG anti-rabbit IgG and FITC rabbit anti-mouse IgG antiserum (Institut Pasteur, Paris, France) were extensively absorbed with human IgG that had been insolubilized with glutaraldehyde. Horseradish peroxidase (HRP)-labeled goat $F(ab')_2$ anti-rabbit IgG antibodies and HRP-labeled rabbit $F(ab')_2$ anti-mouse IgG antibodies were obtained from Institut Pasteur (Paris, France). FITC anti-human IgG, anti-human IgA, anti-human IgM, anti-human Clq and anti-human C3 antisera were obtained from Behringwerke (Marburg lahn, FRG). The anti-C3 antiserum recognizes C3c antigenic determinants expressed on the native C3 molecule and the C3b, C3bi and C3c cleavage fragments of C3. Rabbit anti-human C3d antiserum, which recognizes determinants expressed by native C3, C3b, C3bi, C3dg and C3d, was obtained from the Dutch Red Cross (Amsterdam, The Netherlands). Mouse monoclonal antibody to the C3g antigen [15] was prepared as described. Factor H was purified as described [16] and used as an immunogen to obtain monospecific rabbit antibodies. Affinity purified anti-human C5, anti-human C8, and anti-human C9 rabbit IgG antibodies [13], and rabbit antiserum to the human C3b receptor [17] were raised as described.

Immunofluorescence techiques

Biopsy specimens that had been quick-frozen and stored in liquid nitrogen were sectioned at 3 μm thickness in a Bright's cryostat (Instrument Company Ltd, Huntington, UK). Sections were incubated with phosphate–buffered saline (PBS), pH 7.2, for 20 min at room temperature and overlaid with FITC anti-IgG, anti-IgA, anti-IgM, anti-Clq, anti-C3 antisera or with unlabeled antibodies for 30 min at room temperature. For indirect immunofluorescence (IF), sections were further incubated for 30 min at room temperature with FITC goat anti-rabbit IgG antiserum or with FITC rabbit anti-mouse IgG antiserum. The slides were examined by two investigators with a Leitz microscope (Leitz Inc., Heerbrugg, Switzerland) using epiillumination and an FITC/50 filter. Controls for indirect IF were biopsies incubated with non-immune rabbit IgG and with supernatant from mouse hybridoma NS1 cells.

Conventional and immunoelectron microscopy

Normal part from two nephrectomies for localized renal carcinoma were fixed by perfusing 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4 after catheterization of branches of the renal artery. The specimens were post-fixed in 4% paraformaldehyde during 24 hours. Tissue specimens from diseased kidneys were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 0.5% glutaraldehyde for 4 hours. For conventional electron microscopy, small tissue specimens were post-fixed in 2% OsO_4 for one hour, dehydrated and embedded in epon 812. For immunoelectron microscopy, the tissue specimens that had been fixed in paraformaldehyde were cut into 30 μm sections with a Smith–Farquhar tissue sectioner (Sorvall, Paris, France). Sections from all specimens were incubated with optimal dilutions of anti-C5b-9 antibodies for five hours at room temperature with constant agitation. Additional sections from normal specimens were treated with anti-C3g, anti-C3d, anti-H and anti-C3b receptors antibodies. After washing in cacodylate buffer overnight, the sections were incubated with HRP-labeled $F(ab')_2$ goat anti-rabbit IgG antibody or with HRP-labeled $F(ab')_2$ rabbit anti-mouse IgG antibody. The sections were washed in cacodylate buffer for 36 hours, and peroxidase activity was revealed by reaction with diaminobenzidine and H202 [18, 19]. Sections were post-fixed with OsO_4, dehydrated and flat embedded in epon. Ultrathin sections were cut using a Reichert OmU_2 ultramicrotome (Reichert–Jung, Paris, France) and examined with a Zeiss Em_9 or Em_{10} electron microscope (Carl Zeiss Inc., Oberkochen, FRG). Controls included sections treated with non-immune rabbit IgG or mouse NS1 IgG, and sections treated with HRP-labeled $F(ab')_2$ antibodies alone.

Results

Normal kidneys

Light microscopy and immunofluorescence. All specimens were normal upon light microscopy examination and upon IF

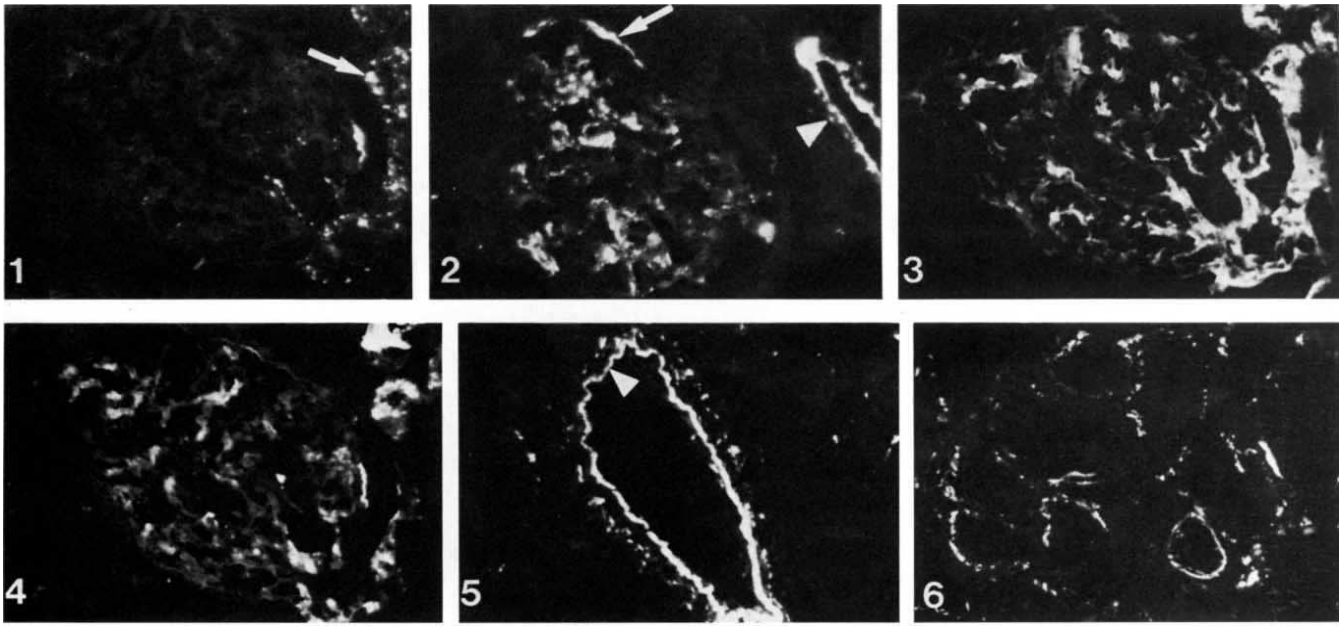

Fig. 1. *Direct immunofluorescence with anti-C3 antiserum.* Morphologically normal biopsy from a patient with isolated microhematuria. A granular staining of juxtaglomerular arterioles (arrow) and of vascular pole is seen. The glomerular tuft is unlabeled; ×273. **Fig. 2.** *Indirect immunofluorescence.* Rabbit anti-human C5b-9 neoantigens antibodies. Morphologically normal biopsy from a patient with isolated microhematuria. Granular staining is seen in mesangial areas, in Bowman's capsule (arrow) and in an arteriolar wall (arrow head); ×273. **Fig. 3.** *Indirect immunofluorescence.* Rabbit anti-human C3d antiserum. Same glomerulus as in Figure 1. Mesangial areas and juxta-glomerular arterioles are labeled; ×273. **Fig. 4.** *Indirect immunofluorescence.* Rabbit anti-human Factor H antiserum. Same glomerulus as in Figure 1. Mesangial areas and juxta-glomerular arterioles are labeled; ×276. **Fig. 5.** *Indirect immunofluorescence.* Rabbit anti-human C5b-9 neoantigens antibodies. Interlobular artery. Same specimen as in Figure 2. Granular labeling is seen outside the spontaneously fluorescent elastic lamina (arrow head) in a perimyocytic pattern; ×276. **Fig. 6.** *Indirect immunofluorescence.* Rabbit anti-human C5b-9 neoantigens antibodies. Same specimen as in Figure 2. Tubular basement membranes are labeled in a ribbon–like or granular pattern; ×178.

with anti-IgG, anti-IgA, anti-IgM, and anti-Clq antisera. A faint and sparse labeling was occasionally seen in glomeruli, and more often in arteriolar walls with anti-C3 antiserum (Fig. 1). In contrast, a fine granular labeling was constantly seen in glomeruli, mainly in mesangial areas, with anti-C5b-9 neoantigens antibodies (Fig. 2), anti-C3g, anti-C3d (Fig. 3), and anti-H antibodies (Fig. 4) in all the specimens that were examined. Occasional segmental labeling of Bowman's capsules was observed with anti-C5b-9 (Fig. 2), anti-C3g, anti-C3d and anti-H antibodies. In arteries and arterioles, anti-C5b-9 (Fig. 5), anti-C3g, anti-C3d, and anti-H antibodies constantly labeled subendothelial spaces and perimyocytic matrices. A similar pattern of glomerular and arteriolar labeling was found with anti-C5, anti-C8, and anti-C9 antibodies. Segmental labeling of some tubular basement membranes (TBM) was seen in a ribbon–like pattern with anti-C5b-9 (Fig. 6), anti-C3g and anti-C3d antibodies but not with anti-H antibody. Anti-C3b receptor antibodies stained all podocytes in glomeruli.

Conventional and immunoperoxidase electron microscopy. The renal tissue was normal by conventional electron microscopy without detectable abnormalities of resident cells and connective matrices, and without detectable immune deposits. SMS and REP were seen in glomerular, tubular, and vascular connective matrices as previously described in normal adult human kidneys. By immunoelectron microscopy, staining with anti-C5b-9, anti-C3g, anti-C3d and anti-H antibodies was found to be localized to SMS and REP within glomerular, vascular and tubular matrices in both morphologically normal specimens that were studied (Figs. 7–12). In glomeruli, labeled REP (Fig. 7) and SMS (Figs. 8–10) appeared isolated or in clusters, distant (Fig. 8), or in close apposition to the plasma membrane of a mesangial cell or of a podocyte [Figs. 7, 9, 10]. When SMS appeared as a closed structure, staining was both circumferential and finely granular inside the structure (Fig. 8). The parts of pedicels and of mesangial cytoplasmic extensions that were in immediate vicinity of REP and SMS were often labeled (Fig. 10). In arteries and arterioles, staining with anti-C5b-9 (Fig. 11), anti-C3g, anti-C3d and anti-H antibodies was observed on SMS and REP in perimyocytic matrices and in the intima. In Bowman's capsules and in TBM, staining with anti-C5b-9 (Fig. 12), anti-C3g and anti-C3d antibodies was seen on clustered REP and vesicles of various sizes. Anti-H antibody stained these structures with less intensity. Anti-C3b receptor antibody diffusely stained the plasma membrane of podocytes and stained some SMS close to the part of the pedicel that was embedded in the GBM (Fig. 9C).

Diseased kidneys

Light microscopy and immunofluorescence. Biopsies were classified into three groups based on the pattern of glomerular staining with anti-C5b-9. In the first group, the pattern of-

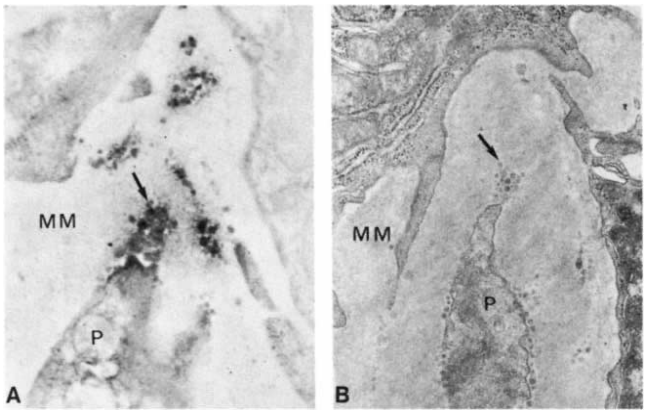

Fig. 7. *Mesangial area.* Morphologically normal tissue by optical, immunofluorescence and standard electron microscopy from a nephrectomy for localized carcinoma. **A.** Indirect immunoperoxidase staining with rabbit anti-human C5b-9 neoantigens antibodies. **B.** Conventional electron microscopy. Clumps of round extracellular particles are embedded in the mesangial matrix (MM). The REP are clearly labeled in **A.** Some clumps of REP (arrow) are located in continuity with a cytoplasmic extension of a podocyte (P); **A** × 19,600; **B** × 12,250.

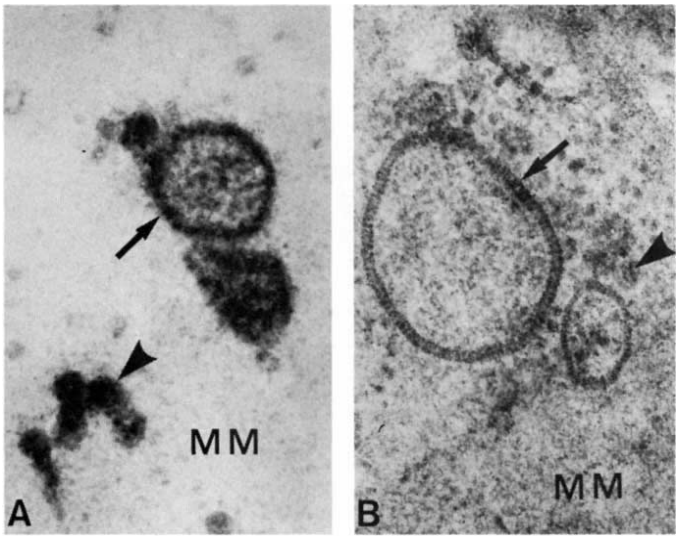

Fig. 8. *Mesangial area.* Same specimen as in Figure 7. **A.** Indirect immunoperoxidase staining with rabbit anti-human C5b-9 neoantigens antibodies. **B.** Conventional electron microscopy. Closed striated membranous structures (SMS) (arrow) and round extracellular particles (REP) (arrow head) embedded in the mesangial matrix (MM). In **A**, staining of the SMS is both circumferential and finely granular inside the structure.REP are also labeled (arrow head); **A** × 53,000; **B** × 57,000.

fluorescence was similar to that observed in morphologically normal kidneys, whether immune deposits were present or absent in the biopsy specimens. In the second group, abundant clusters of C5b-9 neoantigens were seen in enlarged mesangial areas. As in the first group, C5b-9 deposits were independent of the presence or absence of immune deposits. In the third group, most C5b-9 neoantigens were found in the same location as immune deposits. The clinical, morphological and immunohistochemical diagnoses from patients in each group are summarized in Table 1.

Group 1. Twenty–eight patients with the following diagnoses were classified in group 1: MCNS (5), isolated proteinuria with microhematuria (1), DMGN stage I (7), IgA nephropathy (2), Schönlein–Henoch nephropathy (2), DCGN with segmental glomerular necrosis (2), SLE nephropathy type II (1), SLE nephropathy type IV (1), myeloma (2), LCD (1), drug–induced interstitial nephritis (1), acute transplant rejection (2), scleroderma (1). The common feature of this group was that staining with anti-C5b-9 antibodies was restricted to a fine granular labeling in mesangial areas independently of the presence and location of immunoglobulin deposits. There was no labeling with anti-C3 antibodies in 14 of 28 specimens; in the remaining 14, labeling with anti-C3 was sparse and weak. In all specimens, C3d and H antigens were detected in mesangial areas. In specimens from biopsies of patients with DMGN (Fig. 13), SLE and Schönlein–Henoch nephropathy, there was additional diffuse parietal staining with both anti-C3d and anti-H antisera.

Group 2. Twelve patients were included in this group with the following diagnoses: DCGN (3), IgA nephropathy (2), LCD with nodular glomerulosclerosis (1), amyloidosis (2), diabetic glomerulosclerosis (2), IgA nephropathy associated with diabetic glomerulosclerosis (1), Alport's syndrome (1). The essential feature of this group was the presence of abundant clusters of C5b-9 neoantigens in sclerotic glomerular lesions, particularly in enlarged mesangial areas (Fig. 14). Staining for C5b-9 neoantigens was not superimposable with immunoglobulin deposits if these were present. C5b-9 deposits were found in areas of amyloid material. No staining with anti-C5b-9 antibodies was found in areas of extracapillary cell proliferation. Extensive C5b-9 deposition in all specimens contrasted with a weaker and sparser staining with anti-C3 antibodies. The pattern of staining with anti-C3d and anti-H antibodies was similar to that obtained with anti-C5b-9.

Group 3. The twenty–seven patients in this group had immune complex nephritis including: IMGN stage I (2), IMGN stage II (3), IMGN stage III (1), DMGN stage II (1), DMGN stage III (1), SLE nephropathy type IV (7), MPGN type I (9), IgA nephropathy (3). In all specimens, there was extensive parietal and/or mesangial deposition of C5b-9 neoantigens in areas of immunoglobulin and/or C3 deposition. Strong staining for C3d and H antigens was seen in all immune deposits and in extracellular matrices (Fig. 15).

In biopsies from the three groups, granular staining of the intima and of perimyocytic matrices was observed in vessels with anti-C5b-9, anti-C3d, and anti-H antibodies. Additional perimyocytic staining with anti-IgG, and anti-Clq antibodies was seen in most arteries in biopsies from 3 of 9 patients with SLE. Hyalin deposits in arterioles stained for IgM, Clq, C3, C5b-9, C3d and H antigens. Numerous TBM were labeled with anti-C5b-9 and anti-C3d antisera in all pathological specimens that were examined. Staining of TBM was focal and increased with the intensity of the sclerotic process (Fig. 14). In biopsies from patients with SLE, tubulo-interstitial areas that stained for IgG and Clq did not consistently stain with anti-C5b-9 antibod-

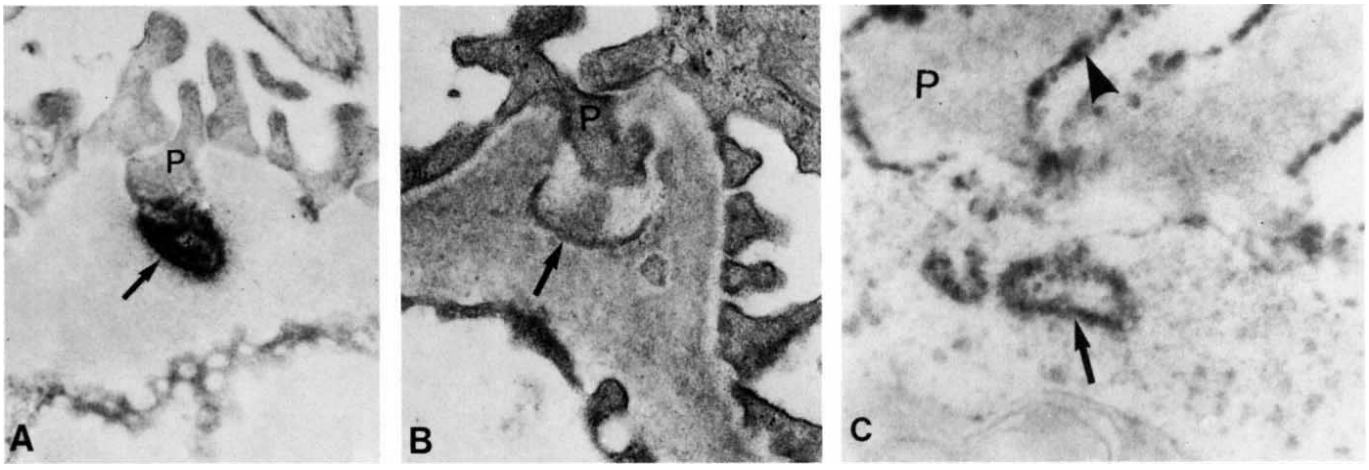

Fig. 9. *Glomerular basement membrane (GBM).* Same specimen as in Figure 7. **A**. Indirect immunoperoxidase staining with rabbit anti-human C5b-9 neoantigens antibodies. **B**. Conventional electron microscopy. Striated membranous structures (SMS) (arrow) underlying an extension of a pedicel (P) is seen within the GBM. In **A**, SMS are strongly labeled. **C**. Indirect immunoperoxidase staining with rabbit anti-human C3b receptor antiserum. The plasma membrane (arrow head) of pedicels (P) and SMS embedded in the GBM (arrow) in the vicinity of a pedicel are labeled; **A** × 26,300; **B** × 36,000; **C** × 65,000.

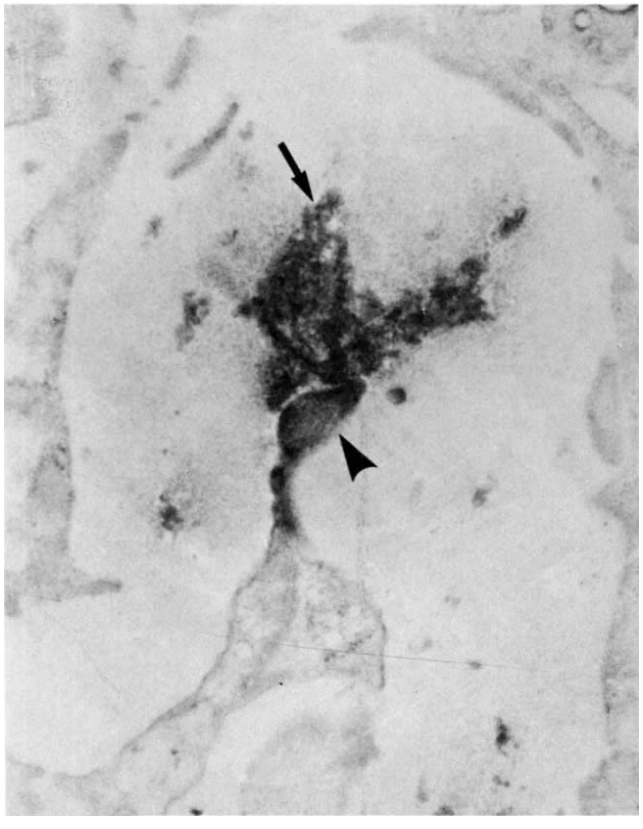

Fig. 10. *Mesangial area.* Indirect immunoperoxidase staining with rabbit anti-human C5b-9 neoantigens antibodies. Electron microscopy. Same specimen as in Figure 7. The distal part of a podocytic extension (arrow head) and striated membranous structure (arrow) are in close vicinity. Both are strongly labeled; × 45,000.

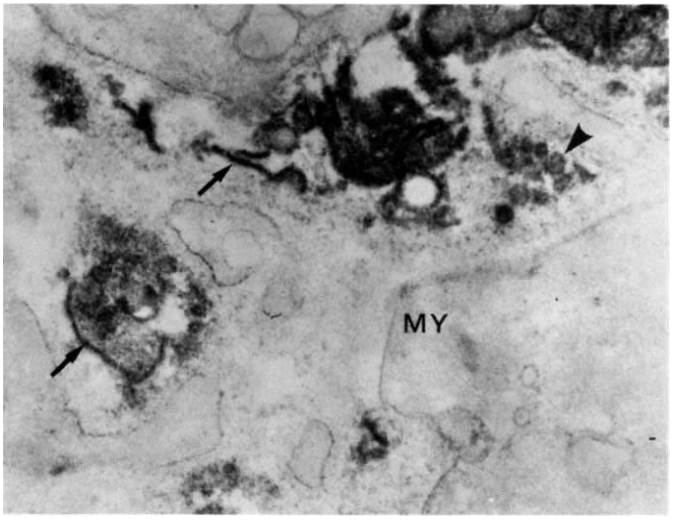

Fig. 11. *Arteriolar wall.* Indirect immunoperoxidase staining with rabbit anti-human C5b-9 neoantigens antibodies. Same specimen as in Figure 7. Striated membranous structures (arrow) and round extracellular particles (arrow head) are labeled. MY: myocyte; ×32,000.

ies. In LCD, focal and segmental staining of TBM with anti-C5b-9 antibodies contrasted with diffuse tubulo-interstitial deposition of kappa or lambda light chain antigens.

Immunoelectron microscopy. Immunoelectron microscopy was performed with anti-C5b-9 neoantigens antibodies on three biopsies that had been classified in group 1 by indirect immunofluorescence (two biopsies from patients with DMGN, stage I; one biopsy from a patient with Schönlein–Henoch nephropathy), one biopsy specimen from group 2 (diabetic glomerulosclerosis with IgA nephropathy) and three biopsy

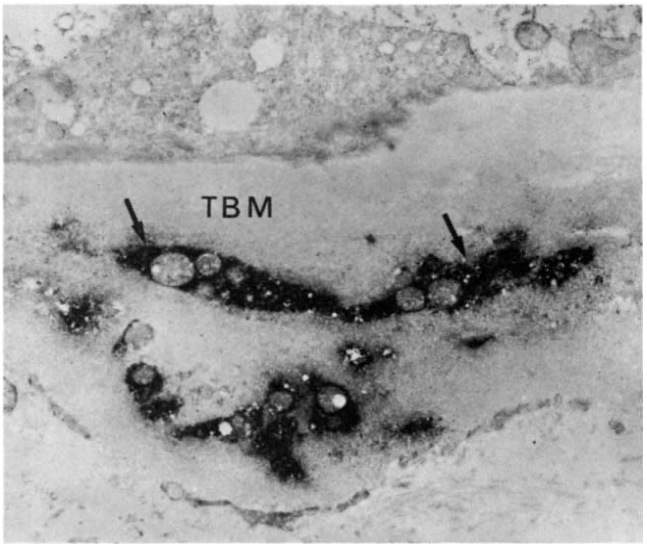

Fig. 12. *Tubular basement membrane (TBM).* Same specimen as in Figure 7. Indirect immunoperoxidase staining with rabbit anti-human C5b-9 neoantigens antibodies. Ribbon–like labeling (arrow) within the TBM surround unlabeled vesicular structures. ×9,000.

specimens from group 3 (two biopsies from patients with MPGN type I and one specimen from a patient with SLE nephritis type IV). In specimens from group 1, strong staining with anti-C5b-9 antibodies was localized to REP and SMS within glomerular, vascular and tubular connective matrices. Immune deposits were not stained or poorly labeled although SMS and REP that were adjacent to or included in some immune deposits were strongly stained (Fig. 16). In the specimen from group 2, the predominant feature was the presence of an increased number of labeled REP and SMS in enlarged connective matrices in areas of glomerulosclerosis (Fig. 17). The increased staining with anti-C5b-9 neoantigens antibodies of TBM was also due to the accumulation of cell remnants in sclerotic TBM (not shown). In the three specimens from group 3, all subendothelial, subepithelial and mesangial immune deposits were strongly and homogeneously labeled with anti-C5b-9 neoantigens antibodies (Fig. 18). SMS and REP in connective matrices were also labeled. No evidence for a lytic process that would have led to necrosis of a resident glomerular cell was 'seen in any of the examined specimens. However, in the three biopsy specimens from group 3, cytoplasmic extensions from endothelial, mesangial, and epithelial cells that were in contact with C5b-9 containing immune deposits, were altered with hyaloplasmic clarification, and sometimes amputated. No ring or cylindrical structures suggestive of inserted C5b-9 were seen.

Controls. All controls were negative.

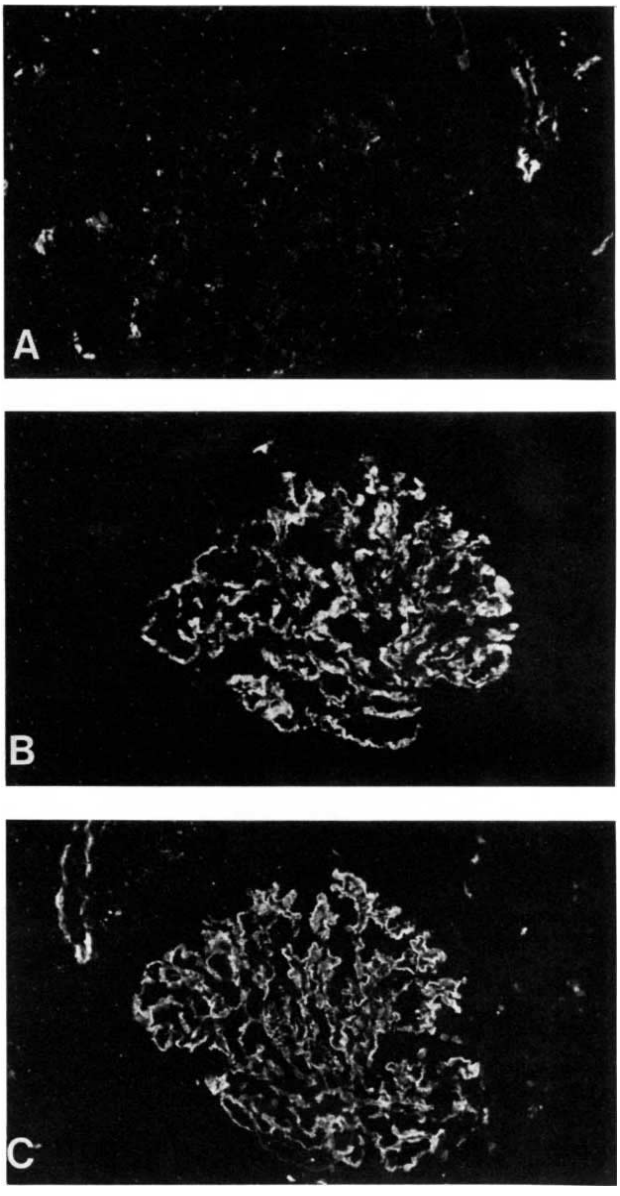

Fig. 13. *Drug–induced membranous nephropathy with diffuse parietal IgG deposits (stage I).* **A.** Staining with anti-C5b-9 neoantigens antibodies. Faint and sparsely granular labeling is seen in the glomerular tuft. **B.** Staining with anti-IgG. **C.** Staining with anti-C3d antibodies. Diffuse granular parietal labeling is seen in the glomerular tufts. Strong staining with anti-C3d antibodies contrasts with the weak staining with anti-C5b-9 and the absence of staining with anti-C3 (data not shown); ×310.

Discussion

In the present study, complement activation products were detected by IF and immunoelectron microscopy in renal biopsies from patients with various types of nephropathies, and also in adult human kidney specimens that were normal upon light microscopy, routine IF examination, and conventional electron microscopy. By immunoelectron microscopy, C5b-9 neoantigens of the terminal complement sequence were found on striated membranous structures (SMS) and round extracellular particles (REP) within connective matrices in all kidneys that were examined, either normal or diseased. In diseased kidneys, C5b-9 neoantigens were additionally found within large immune deposits.

Table 1. Pattern of staining with anti-IgG, anti-IgA, anti-IgM, anti-C3, anti-C3d, anti-H and anti-C5b-9 in 67 diseased kidneys

Disease category		Pattern of glomerular staining[a]						
		IgG	IgA	IgM	C3	C3d	H	C5b-9
Group 1								
Minimal change nephrotic syndrome (MCNS)	(2)[b]	0	0	0	0	M+	M+	M+
	(1)	0	0	M+	0	MP+	M+	M+
	(1)	0	0	M+	M+	M+	M+	M+
	(1)	0	0	0	M+	M+	M+	M+
Isolated microhematuria	(1)	0	0	0	MP+	M+	M+	M+
Drug–induced membranous nephropathy (DMGN) stage I	(5)	P+	0	0	0	P+	P+	M+
	(2)	P+	0	0	P+	P+	P+	M+
IgA nephropathy	(2)	0	M+	M+	M+	M+	M+	M+
Schönlein–Henoch nephropathy	(2)	MP+	M+	M+	MP+	MP+	MP+	M+
Diffuse crescentic glomerulonephritis (DCGN)	(1)	0	0	0	0	M+	M+	M+
	(1)	+ segm	0	0	MP+	M+	M+	M+
Scleroderma with thrombotic microangiopathy	(1)	0	0	0	MP+	M+	M+	M+
SLE nephropathy, type II	(1)	M++	M+	M±	M+	MP+	MP+	M+
type IV	(1)	+ segm	+ segm	+segm	M+	MP+	MP+	M+
Myeloma	(1)	0	0	0	0	M+	0	M+
	(1)	0	0	0	M+	M+	M+	M+
Light chain disease (LCD)	(1)	0	0	0	0	M±	M±	M+
Drug–induced acute interstitial nephritis	(1)	0	0	0	0	M+	M+	M+
Acute transplant rejection	(2)	0	0	0	0	M+	M+	M+
Group 2								
Diffuse crescentic glomerulonephritis	(1)	+ segm	P+	0	P+	P+	P+	M++
	(1)	++ segm	0	0	M++	P+	P+	M++
	(1)	+ segm	0	0	+ segm	+ segm	+ segm	M++
IgA nephropathy	(1)	0	M+	0	M+	P+	P+	M+++
	(1)	0	M+	P±	MP+	MP+	M+	M++
Light chain disease	(1)	0	0	0	M+	M++	M++	M+++
Amyloidosis	(1)	+[c]	0	0	M+	M+	M+	M+++[c]
	(1)	+[c]	+[c]	+[c]	0	+[c]	++[c]	M+++[c]
Diabetic glomerulosclerosis	(2)	P±	0	0	M++	MP++	MP+	M++
IgA nephropathy associated with diabetic glomerulosclerosis	(1)	0	M+	+ segm	M+	MP++	MP+	M+++
Alport's syndrome	(1)	0	0	P±	M+	P+	P+	M++
Group 3								
Idiopathic membranous nephropathy stage I	(2)	P+	P±	0	P+	P+	P+	P+
stage II	(2)	P++	0	0	P+	P++	P++	P++
	(1)	P++	0	0	0	P++	P++	P++
stage III	(1)	P++	0	P±	P+	P++	P++	P++
Drug–induced membranous nephropathy stage II	(1)	P++	0	P+	P+	P+	P+	P+
stage III	(1)	P++	0	0	P++	P++	P++	P++

Table 1. Continued

Disease category		Pattern of glomerular staining[a]						
		IgG	IgA	IgM	C3	C3d	H	C5b-9
Membranoproliferative glomerulonephritis	(2)	0	0	0	MP++	P++	P++	MP++
	(4)	MP++	0	0	MP++	MP+	MP+	MP++
	(1)	P±	MP++	0	MP++	MP+	M+	MP++
	(1)	+ segm	+ segm	+ segm	+ segm	P+	+ segm	MP+
	(1)	± segm	0	+ segm	+ segm	P+	P+	P+
IgA nephropathy	(1)	M±	M+	M±	MP+	MP+	MP+	MP+
	(2)	M±	M+	M±	MP+	P+	P+	M++
SLE nephropathy, type IV	(6)	MP++	MP+	MP±	MP++	MP++	MP++	MP++
	(1)	MP++	MP+	MP++	MP++	MP+++	MP++	MP++

[a] Abbreviations are 0, no staining; segm, segmental, that is, affecting only part of the glomerulus; foc, focal, that is, affecting only some glomeruli; M, mesangial; P, parietal; MP, mesangial and parietal. Intensity was graded from ± to +++.
[b] Parenthesis indicates the number of patients in a diagnostic category with similar immunohistochemical findings in all patients studied.
[c] The topography of immune and complement deposits is superimposable with that of amyloid deposits

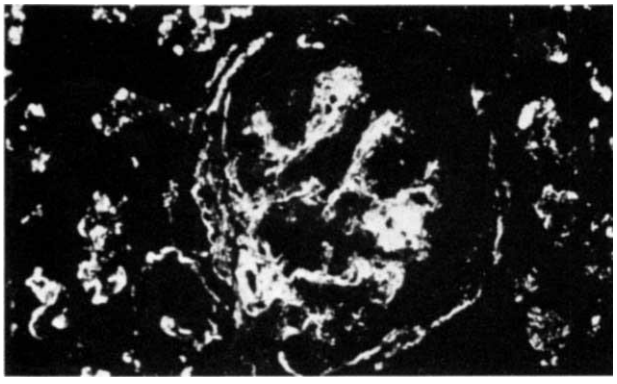

Fig. 14. *Diabetic glomerulosclerosis.* Staining with anti-C9 neoantigens antibodies. Extensive C5b-9 deposits are seen in nodular mesangial areas; ×310.

Morphologically normal adult human kidney specimens included six renal biopsies from patients from 19 to 40 years old with minimal urinary abnormalities, and morphologically normal tissue from nephrectomies for localized carcinoma that were performed in two patients of 52 and 60 years old. Immunoelectron microscopy was performed on the nephrectomy specimens. Such material has previously been used to investigate the presence of various antigens in normal renal tissue [17, 20, 22]. By indirect immunofluorescence, staining for C5b-9 neoantigens, C3g and C3d antigens was observed within glomerular, arteriolar, and tubular connective matrices. The finding of C5b-9 neoantigens in these locations was in agreement with the previous observation of poly-C9 antigen in matrices of normal human kidney [8] and of C5b-9 neoantigens in renal connective matrices of normal rats [9]. Staining was also observed with anti-native C5 and anti-C8 antibodies, indicating the presence of true terminal complexes of the complement sequence. Complement activation in connective matrices is further supported by the presence of Factor H, which recognizes C3b, and of the terminal C3dg cleavage fragment of C3, as apparent from the presence of both C3g and C3d antigens in the absence of C3c antigen [15]. Occasional staining of connective matrices with anti-C3 antiserum did not correlate with the constant finding of C3g, C3d, H, and C5b-9 antigens. By immunoelectron microscopy, the sites of complement activation were identified as SMS, REP, and vesicular structures of various sizes embedded in connective matrices. SMS, REP, and vesicular structures have been observed in connective matrices of normal [23–26] and diseased [23–29] human kidney and in connective matrices of myocardium [30], seminiferous tubules [31] and Bruch's membrane [32]. Their presence in morphologically normal renal specimens does not imply the presence of subclinical renal disease since SMS and REP have been routinely observed in immediate renal graft biopsies [23–26].

That SMS and REP represent fragmented cellular processes is suggested by their ultrastructural appearance [23–27, 30] and location. The finding in this study that those SMS, which are located in the subepithelial part of the GBM, stained for C3b receptor antigen, a specific marker for podocytic membranes [17], and further suggests that SMS are plasma membranes detached from adjacent cells. Terminal C5b-9 complexes could form on primarily shedded membrane remnants. Thus, killed human kidney cells [33], heart mitochondrial membranes in ischemic myocardial cells [34], and cytoskeletal intermediate filaments in cultured human embryonal fibroblasts [35] activate the complement system in the absence of antibody. Alternatively, attachment of C5b-9 to cell membranes could lead to secondary shedding of the afflicted membrane areas. The latter possibility is suggested by the present finding of C5b-9 neoantigens on podocytic and mesangial cytoplasm in immediate vicinity of SMS and REP. In either case, triggering of the complement cascade on cell membranes and/or remnants might be due to reduction or loss of regulatory membrane factors that normally inhibit activation of the complement system on autologous cells [36]. The presence of Factor H and of C5b-9 neoantigens in similar locations indicates that activation and control of complement coexist on cell membranes, although

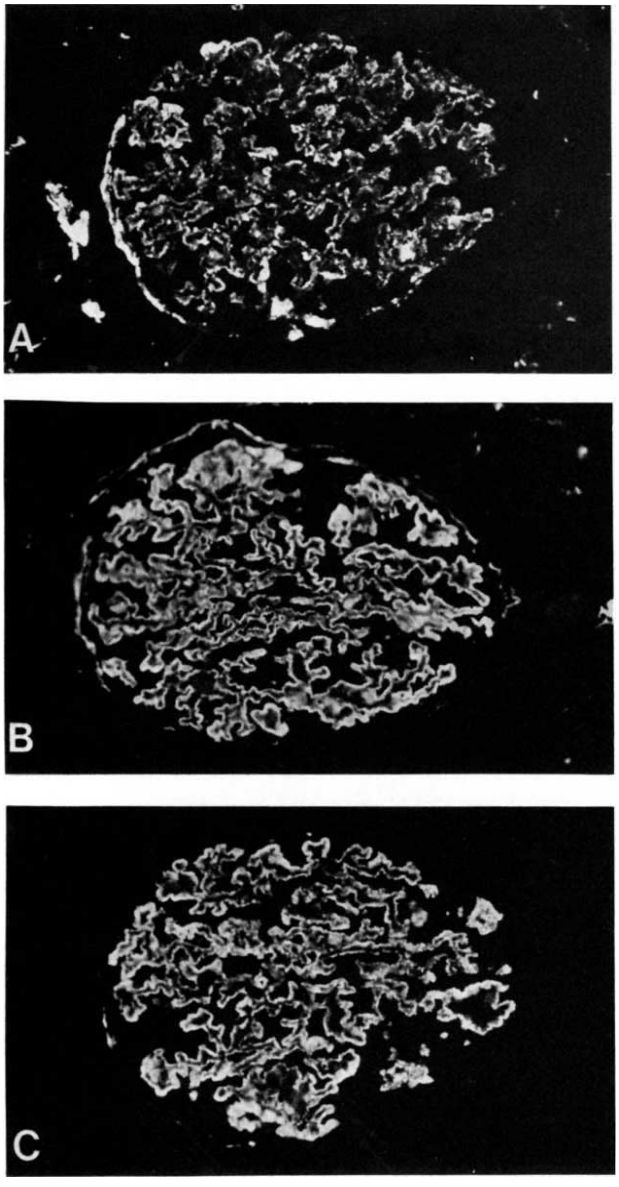

Fig. 15. *Idiopathic membranous nephropathy, stage II.* Serial sections of the same glomerulus stained with anti-C5b-9 neoantigens antibodies (**A**), anti-C3d antibodies (**B**) and anti-H antibodies (**C**). A diffuse granular parietal pattern of staining is seen with each of the three antibodies, similar to that observed with anti-IgG and anti-C3 (data not shown); ×310.

activation may escape control and result in formation of C5b-9 complexes. C5b-9 complexes are remarkably stable structures [1] and probably remain trapped in connective matrices, inacessible to inflammatory cells, over long periods of time. Their generation in normal tissues may be of no immunopathological consequence because of the shielded environment in which the reaction occurs.

Kidney biopsies from 67 individual patients representing a large panel of clinical and immunohistochemical diagnoses were also investigated for the presence of C5b-9 neoantigens by IF.

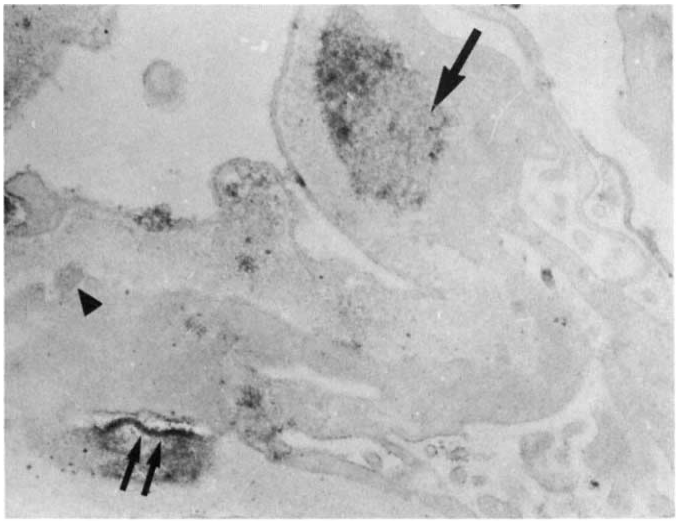

Fig. 16. *Electron micrograph of a glomerulus from a patient with drug–induced membranous nephropathy stained by immunoperoxidase with anti-C5b-9 neoantigens antibodies.* Subepithelial deposits are not (arrow head) or poorly and heterogeneously (arrow) labeled. A striated membranous structure adjacent to a subepithelial deposit is strongly stained (double arrow); ×11,000.

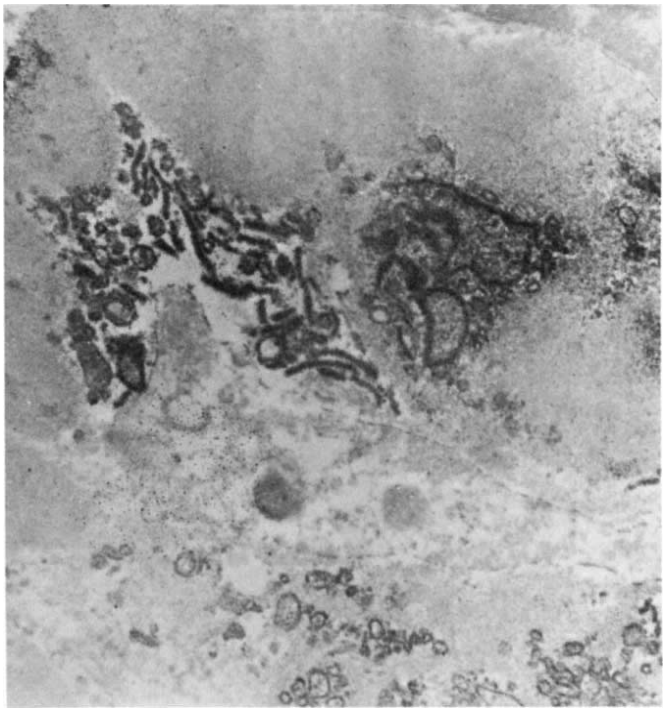

Fig. 17. *Electron micrograph of a glomerulus from a patient with diabetic glomerulosclerosis stained by immunoperoxidase with anti-C5b-9 neoantigens antibodies.* The enlarged glomerular basement membrane and mesangial matrix contain numerous striated membranous structure (SMS), round extracellular particles (REP) and other cell remnants. SMS, REP and cell remnants are strongly labeled; ×35,000.

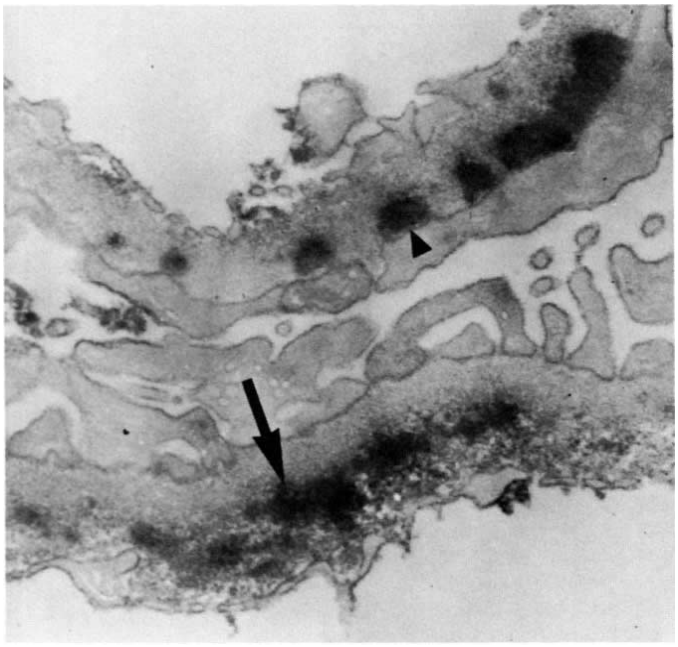

Fig. 18. *Electron micrograph of capillary walls in a glomerulus from a patient with SLE nephropathy type IV, stained by immunoperoxidase with anti-C5b-9 neoantigens antibodies.* All subendothelial (arrow) and subepithelial (arrow head) immune deposits are strongly and homogeneously labeled; ×25,500.

Seven biopsies were studied by immunoelectron microscopy The pattern of glomerular staining with anti-C5b-9 antibodies as discerned by indirect IF permitted classification of the biopsies into three categories. In the first group, staining for C5b-9 neoantigens was similar with that of normal kidney. By immunoelectron microscopy, C5b-9 neoantigens were localized to SMS and REP. This group included 14 biopsies without immunoglobulin deposits and 13 biopsies with small or segmental immune deposits, of which seven showed an early stage of DMGN. In these cases, deposition of C5b-9, which occurred in the absence of immunoglobulin deposits or in a different location from that of immunoglobulins, was unlikely to represent the effector mechanism by which immune deposits were pathogenic. C5b-9 neoantigens, however, could be present in immune deposits in quantities below the threshold of their detection. The presence of C3d and H antigens in the same location as that of parietal IgG in DMGN suggest that complement may be activated *in situ*, but that effective regulation prevents recruitement of the terminal effector sequence.

In the second group of biopsies, anti-C5b-9 antibodies extensively stained enlarged mesangial areas in the presence or absence of labeled glomerular immune deposits. The strong mesangial staining derived from increased amounts of labeled SMS and REP in glomerular sclerotic areas, as revealed by immunoelectron microscopy. The increased amount of cell remnants in sclerotic matrices [23–25, 27, 28] account for the previous observation of abundant deposits of "poly-C9" antigen in diabetic glomerulosclerosis, hypertensive nephrosclerosis, and end–stage kidneys [8]. That cell remnants also stain for C3d and H explains the increase in staining intensities for these antigens in glomerular and tubular sclerotic matrices in group 2 as compared with group 1.

The third group comprised biopsies with extensive subepithelial, subendothelial and/or mesangial immune deposits. In all biopsies from this group, the pattern of staining with anti-C5b-9 antibodies was similar to that of immunoglobulins. Upon immunoelectron microscopic examination, there was strong and homogeneous staining of all immune deposits in addition to staining of cell remnants. Immune deposits also stained for C3d and H antigens, indicating that activation and regulation of complement occur on large immune deposits. C5b-9 deposition probably ensues when activation processes escape control. Staining for C5b-9 that was observed in subepithelial deposits only at late stages of DMGN and only at late stages of *de novo* MGN in transplant recipients [37] indicates local rearrangement of immune deposits [38], and suggests that C5b-9 is unrelated to the pathogenesis of proteinuria. In this respect, the pathogenesis of proteinuria in DMGN stage 1 would differ from that of an experimental model of MGN in rabbits in which proteinuria is dependent on normal C6 activity [11].

Evaluation of immunohistochemical data on renal biopsies stained with anti-C5b-9 antibodies requires careful interpretation; sparse and granular labeling in connective matrices should not be regarded as abnormal, since C5b-9 is normally found on cell remnants that are embedded in connective matrices. Conversely, the presence of C5b-9 neoantigens is of pathological significance when the antigens are found within immune deposits and/or are present in abundant clusters in sclerotic matrices because of the increased density of labeled cell remnants in the latter circumstances. The pathological occurence of C5b-9 neoantigens does not strictly correlate with positivity for C3, that is, some biopsies were negative for C3 but positive for C5b-9, which may derive from the extreme stability of C5b-9 as opposed to C3 antigens.

Complement–mediated damage to a membrane results from the insertion in the lipid bilayer of C5b-9 complexes to create hydrophilic transmembrane channels [1]. Although erythrocytes are effectively lyzed by small amounts of C5b-9, nucleated cells are more resistant to membrane attack by complement. Furthermore, cell membranes are relatively resistant to lysis by autologous, as compared with heterologous complement [39]. Although C5b-9 has been found in this and in previous [7, 8] studies to be present in various types of renal lesions, its direct role as a pathogenic mediator of renal injury remains speculative. The present observation of C5b-9 neoantigens on cell remnants in normal human adult kidney could suggest that complement plays a physiological role in the normal shedding process of plasma membranes from aging cells. Recognition of the diversity in the presence and localization of C5b-9 neoantigens in diseased kidneys is relevant to the understanding of the role of the effector sequence of complement in the pathogenesis of immune and non-immune renal diseases.

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