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Research Article

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The Immunohistopathology of Glomerular Antigens

THE GLOMERULAR BASEMENT MEMBRANE, COLLAGEN, AND ACTOMYOSIN ANTIGENS IN NORMAL AND DISEASED KIDNEYS

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ABSTRACT The immunofluorescent localization of antisera to human glomerular basement membrane (GBM), collagen, and smooth muscle actomyosin was examined in 15 specimens of normal renal tissue and 98 specimens from patients with renal disease. The anti-GBM and anticollagen antisera normally localize to GBM, while antiactomyosin localizes to the mesangium. Diabetic nephropathy revealed a striking expansion of mesangial material reacting with antiactomyosin. In contrast, the expanded mesangium in membranoproliferative glomerulonephritis did not react with antiactomyosin, and the GBM localization of anti-GBM and anticollagen sera was similarly lost. The thickened GBM in diabetes mellitus and membranous nephropathy reacted with anti-GBM and anticollagen, but with accentuation of staining on the inner aspect of the GBM. In proliferative glomerulonephritis there was a moderate increase in the distribution of actomyosin. Glomerular sclerosis and hyalinization in all diseases studied was accompanied by a loss of immunofluorescent staining for all glomerular antigens, including collagen.

INTRODUCTION

Immunopathologic studies of the human kidney are generally utilized to demonstrate deposition of serum proteins or foreign antigens. Normal structural glomerular antigens have also been demonstrated by immunofluorescence: antigenic components of the glomerular basement membrane (GBM)[1] react with heterologous nephrotoxic anti-GBM serum, anti-GBM antibodies of Goodpasture's syndrome (1), and antibody to solublized skin collagen (2). The glomerular mesangium has been shown to react with antibody to smooth muscle actomyosin (3).

In the present studies the distribution and amounts of glomerular antigens in the diseased kidney are evaluated by immunopathologic methods using specific antisera to GBM, collagen, and actomyosin. Special attention has been directed to those diseases in which specific alterations in glomerular architecture are usually found, but where the relationship of these alterations to the normal glomerular components is unknown: the GBM thickening of diabetes mellitus and membranoproliferative glomerulonephritis, the replacement of normal glomerular architecture in focal sclerosis (segmental hyalinization), and end-stage glomerular obsolescence (diffuse hyalinization).

METHODS

Antigens

Actomyosin was isolated from a human uterus weighing 160 g obtained at autopsy from a 27-yr-old woman 16 h after accidental death by asphyxiation. The patient had aborted 2 wk previously in the 20th wk of pregnancy. The isolation was performed at 4°C after the methods of Becker (3) and Finck (4). Initially, the washed and homogenized myometrium was extracted for 18 h in 400 ml of a buffer (0.3 M KCl, 0.17 M K_2HPO_4, 0.001 M EDTA, pH 8.5) of calculated ionic strength (μ) = 0.8, to which 0.08% ATP[2] was added. The brei was filtered through Miracloth[3] and the protein precipitated by dilution to $\mu = 0.03$, collected by centrifugation at 3,300 *g* for 15 min, and washed twice. The precipitate was solubilized in 200 ml of a solution of $\mu = 0.57$ (0.13 M KCl, 0.095 M KH_2PO_4, 0.075 M K_2HPO_4, 0.001 M EDTA, pH 6.5) by dispersion with a Pasteur pipette and slow stirring for 2 h. This and subsequent solutions were clarified by centrifugation at 37,000 *g* for 90 min. The protein was again precipitated by

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[1] *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; μ, calculated ionic strength; SDS, sodium dodecyl sulfate; TBM, tubular basement membrane.

[2] Sigma Chemical Co., St. Louis, Mo.

[3] Calbiochem, San Diego, Calif.

dilution to $\mu = 0.03$, solubilized in $\mu = 1.15$ buffer (0.81 M KCl, 0.11 M KH_2PO_4, 0.1 M K_2HPO_4, 0.001 M EDTA, pH 6.5), and precipitated at $\mu = 0.3$. Solubilization in 90 ml of the $\mu = 1.15$ buffer and precipitation at $\mu = 0.3$ were repeated. Next, the protein was twice solubilized at $\mu = 1.15$ and precipitated by dilution to $\mu = 0.1$. The protein was dissolved in 40 ml of 0.02 M Tris-HCl buffer containing 0.5 M KCl, pH 7.4, stabilized by the addition of 40 ml of 5.0 M LiCl, pH 7.0, and precipitated at 4°C with $(NH_4)_2SO_4$ at 39% saturation (vol/vol). After the precipitate was washed, solubilized in 10 ml of the 0.5 M KCl solution, and dialyzed, 3 ml (approximately 10 mg by OD at 280 nm [4] was applied by reverse flow to a 1.5×100-cm closed jacketed column of Sephadex G-200 Superfine[4] packed on siliconized 6-mm glass beads (5), running at 7 ml/h at 4°C. The column was supported on a Teflon mesh[4] to avoid precipitation of the protein during loading. The protein emerged at the void volume in a single peak. It was preserved in 50% glycerol and used for immunization of rabbits and immunodiffusion tests. Portions of the unchromatographed protein were used for ATPase assay (6) and released 3.6 μg P/mg protein/30 min. Polyacrylamide gel (7.5%) chromatography in sodium dodecyl sulfate (SDS) (7) was performed by Ms. Juille Johnson in the laboratories of Drs. M. Han and E. Benson (Departments of Laboratory Medicine and Pathology, University of Minnesota Medical School). The gel electrophoresis pattern of our actomysin preparation (in SDS) was compared with purified samples of rabbit skeletal muscle actin and myosin and revealed multiple bands similar to those of actin, as well as two bands in the region of myosin.

Collagen was isolated from skin samples from neonatal autopsies. Acid-soluble collagen was extracted at 4°C in dilute acetic acid (pH 3.2) and isolated by neutral precipitation and acid solubilization three times, as described by Rothbard and Watson (2). The collagen was lyophilized and dissolved in 5 M guanidine-HCl and dialyzed against 8 M urea for immunization.

Human GBM was isolated from two normal kidneys at autopsy within 5 h of death as described previously in this laboratory (8).

Immunologic techniques

Fluorescein isothiocyanate[5] (FITC) labeling of the IgG fraction of antisera was performed and the optimally labeled fractions (fluorescein: protein ratio $6\text{–}10 \times 10^{-3}$) were obtained by DEAE[6] chromatography by using the method of Cebra and Goldstein (9). Direct and indirect immunofluorescent staining was performed as described previously in this laboratory (10).

Tissues were placed on moistened Onkosponge no. 1[7] and snap frozen in isopentane prechilled in liquid N_2 and stored at -70°C until sectioning at 2–4 μm on a Lipshaw 1500 Cryotome cryostat (Lipshaw Mfg. Co., Detroit, Mich.) or at 1–2 μm on a Harris MS-100 Microtome cryostat (Harris Mfg. Co., Cambridge, Mass.). No deterioration or changes in the fluorescent staining reactions reported here were noted when restaining freshly cut tissue stored up to 20 mo in isopentane at -70°C. Tissues were examined with a Zeiss fluorescent or Ultraphot III microscope with a Zeiss FITC interference filter and an OG-4 barrier filter (Carl Zeiss, Inc., New York).

Immunodiffusion was performed as previously reported (10). Actomyosin was studied at 4°C on 1% Noble agar in 0.5 M KCl, 0.02 M Tris-HCl, pH 7.4; antigen was placed in wells 5 mm in diameter and the antiactomyosin antisera in 1.3-mm wells and allowed to diffuse for 3–7 days at 4°C. Immunodiffusion of acid-soluble collagen was attempted in acetic acid, guanidine, urea (2), and collagenase solutions, on agarose and Noble agars, and in glycine and Veronal buffers.

Other studies

Collagenase[8] digestion of normal human kidney frozen tissue sections was described by Rothbard and Watson (11) before immunofluorescent staining. Thioflavine T stain for amyloid was performed on frozen sections after formalin fixation (12). Tissue targets for immunofluorescence included the following: frozen sections of a biopsy of gravid human term uterus; normal skin from an 11-yr-old boy with ulcerative colitis and purpura; a normal bowel biopsy; normal human kidney obtained at autopsy within 4 h of death in an accident victim. The buffy coat of human blood smears was aid dried, fixed in absolute alcohol-ether (1:1), and stained for immunofluorescence.

Antisera

Antiactomyosin (*antiserum to actomyosin*). Actomyosin, 1 mg in 50% glycerol-0.5 M KCl solution in complete Freund's adjuvant, was injected subcutaneously into each rabbit weekly for 5 wk. These rabbits were then boosted every 2 wk and antisera harvested for up to 8 more wk. The strongest pool of antiserum was absorbed with normal human serum on a Sepharose 4B[9] immunoabsorbent column (13). This antiserum showed two precipitin lines by immunodiffusion against the actomyosin used for immunization. These lines, at differing dilutions, formed lines of identity with those formed by antiactomyosin antiserum provided by Dr. C. G. Becker (N. Y. Hospital, Cornell Medical Center). The immunodiffusion reactions were abolished by two absorptions of our antiserum with precipitated uterine actomyosin (approximately 5 mg/ml of undiluted antiserum). There was no reaction between this antiserum and normal human serum at dilutions of both up to 1:512.

Antiactomyosin stained frozen sections of a gravid (term) human uterus strongly, as well as bowel wall muscularis and smears of platelets, segmented neutrophils, and some mononuclear cells. Glomerular mesangial staining in the normal human kidney and staining of gravid uterine muscle were completely inhibited on indirect immunofluorescence after two absorptions with precipitated actomyosin. Minimal reduction of mesangial staining was produced by exhaustive absorption of the antiserum with lyophilized GBM (25 mg/ml undiluted antiserum). No inhibition was seen after two consecutive absorptions with collagen (12.5 mg/ml undiluted antiserum, performed twice).

Anticollagen (*antiserum to collagen*). Collagen, 15 mg, dissolved in 8 M urea, was used for each immunization in complete Freund's adjuvant. Seven rabbits and one goat were immunized weekly in multiple subcutaneous sites for 4 wk and then biweekly for 16 wk with weekly test bleeds. Antiserum localizing by indirect immunofluorescence to

[4] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

[5] BBL, BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md.

[6] Bio-Rad Laboratories, Richmond, Calif.

[7] Histamed, Patterson, N. J.

[8] Collagenase, CLSPA, Worthington Biochemical Corp., Freehold, N. J.

[9] Pharmacia Fine Chemicals, Inc.

human GBM appeared in two of seven rabbits; of the two rabbits developing antibody, the stronger was used for subsequent studies. The IgG fraction (10 mg/ml) of this antiserum did not react on immunodiffusion against solubilized collagen nor with normal human serum. None of the remaining rabbits developed detectable antibody after 4 mo of immunization. The goat developed weak antibody, staining the glomerulus diffusely without specific GBM localization.

Anticollagen localized on sections of human dermis to bundles of fibrillar material which extended into the rete pegs, as well as to the mesangium of the glomerulus. All fluorescent reactions were inhibited by prior absorption of the antiserum with lyophilized collagen (12.5 mg/ml of undiluted antiserum, performed twice), but not by similar absorption with GBM or actomyosin.

A sample of rabbit antiserum to 0.5 M acetic acid-soluble NaCl-precipitable human collagen (14) was also kindly supplied by Dr. Dov Michaeli (Department of Biochemistry, University of California, San Francisco). By indirect immunofluorescence this antiserum stained the glomerulus more diffusely than did ours, without apparently specific GBM localization. However, other tissue staining reactions by indirect immunofluorescence were similar though somewhat stronger than ours and were abolished by two absorptions with 0.7 mg of our lyophilized collagen preparation per ml of a 1:64 dilution of the antiserum.

Anti-GBM (antiserum to GBM). 1 mg of lyophilized GBM was injected in complete Freund's adjuvant into a rabbit by using multiple subcutaneous sites; animals were boosted 5 wk and 8 mo later and bled 1 wk later. This antiserum localized to human GBM and tubular basement membranes (TBM) by indirect and direct immunofluorescence. Its absorption with lyophilized GBM (12.5 mg/ml of undiluted antiserum, performed twice) completely inhibited its localization to basement membranes by indirect immunofluorescence. Similar absorption of unlabeled antiserum with lyophilized collagen, actomyosin, or normal human serum did not alter the fluorescent staining reaction. Two absorptions of the FITC-labeled antiserum with actomyosin did not inhibit its staining of the GBM. The antiserum did not react on immunodiffusion against normal human serum.

Human (Goodpasture's) anti-GBM antibody was eluted from the nephrectomy specimen of a patient with pulmonary hemorrhage, rapidly progressive nephritis, and linear IgG deposition along the GBM seen by immunofluorescence (1). The Goodpasture's antibody localized only to GBM by indirect immunofluorescence.

Patient material

113 percutaneous and open kidney biopsies and nephrectomy specimens from 109 patients admitted to the Pediatric, Medical and Surgical services of the University of Minnesota Hospitals were examined in this study: normals included biopsies from 11 kidney transplant donors and four autopsy specimens, obtained within 4 h of accidental death.

Tissues from the following diseases were studied (Table I): membranous nephropathy, 16 patients (including six

TABLE I

Alterations in Glomerular Antigens in Renal Disease

		GBM fluorescence using antibodies to GBM and collagen			Mesangial fluorescence using antibody to actomyosin			
							Expanded	
Disease	No. of tissues examined	Normal	Absent or trace	Thickened*	Normal	Absent or trace	Mildly	Markedly
Normal	15	15			15			
Membranoproliferative glomerulonephritis	19		19			17		
Membranous nephropathy	16	2		14	8		7	
Diabetes mellitus								
Onset	1	1			1			
Early	2			2				2
Late	16	1		10				9
End-stage	5		4	1		5		
Proliferative nephritis	11	11					11	
Nephrotic syndrome	11	11			8		3	
Glomerular hypertrophy	2	1		1	1		1	
Amyloid nephropathy	3	3			2			
End-stage kidney	12							
Residual glomeruli		8			8			
Hyalinized glomeruli			12			12		

Predominant findings for fluorescent localization of antisera in renal diseases. GBM staining ***absent or trace*** indicates very thin or absent linear staining of capillary loops. ***Thickened*** GBM is judged at its narrowest appearance among different planes of focus. Mesangial staining ***absent or trace*** indicates absence of, or only small residual patches of, mesangial staining. ***Mildly expanded*** mesangial staining with antiactomyosin indicates loss of the limited mesangial distribution and extension to the periphery and often around glomerular loops. ***Markedly expanded*** mesangial staining with antiactomyasin indicates that the widened mesangium is stained to the full extent of that observed by light microscopy in these tissues.

The number of tissues examined exceeds the number for which results are given, due to inadequate tissue quality or autofluorescence precluding satisfactory evaluation. More than three glomeruli were evaluated for each result given, except that two glomeruli were present in two sections from membranoproliferative glomerulonephritis, one section from nephrotic syndrome, and one section from poststreptococcal glomerulonephritis.

* The GBM had a laminated appearance, with the inner layer more densely stained in nine of 14 with membranous nephropathy, six of 10 with late diabetes, and one with end-stage diabetes.

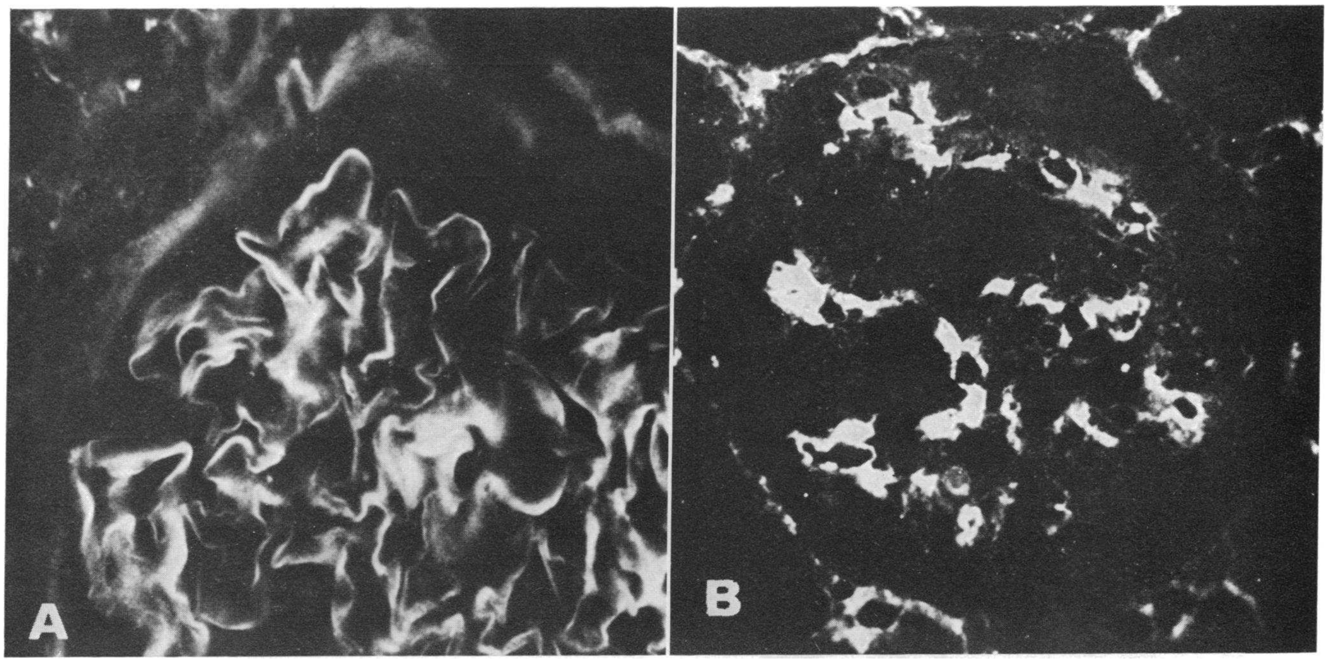

FIGURE 1 Immunofluorescent localization of glomerular antigens in the normal glomerulus. (A) Anti-GBM localization to the GBM and Bowman's capsule. The mesangium is not stained (anticollagen staining is similar, not illustrated). (B) Antiactomyosin staining of the mesangium. Sections 2 μm, all photographs 1,000 ×.

with systemic lupus erythematosus); membranoproliferative glomerulonephritis, 19 specimens from 16 patients; diabetes mellitus, 24 patients; nephrotic syndrome, including three nil-lesion, one steroid responsive with focal sclerosis, five steroid resistant with focal sclerosis, and two congenital (Finnish type); proliferative nephritis including three poststreptococcal, three anaphylactoid purpura nephritis, four focal mesangial proliferative nephritis, and one rapidly progressive nephritis; glomerular hypertrophy, one patient with cyanotic congenital heart disease and proteinuria (15) and one child with renal failure due to bilateral renal hypoplasia with oligonephronia (16); amyloid nephropathy, three specimens from two patients. End-stage renal disease included two obstructive pyelonephritis, one congenital dysplasia, three medullary cystic disease, one cystinosis, and five unknown causes.

Immunopathologic analyses

Tissues were evaluated by the senior author (J. I. S.), and all significant deviations from normal patterns were read blindly by at least one of the other authors. More than three glomeruli were present per section except where indicated in Table I. A complete set of stained sections from the same normal tissue accompanied each group of stains for comparison. The intensity of glomerular staining was judged relative to nonglomerular structures: anti-GBM staining of GBM was compared to TBM (approximately equal in normal kidney); anticollagen staining of GBM was compared to interstitial staining (slightly less intense in normal kidney); antiactomyosin staining of the mesangium was compared to peritubular capillary staining (approximately equal in normal kidney). However, because of the difficulty in judging intensity of fluorescence, abnormalities were primarily judged by distribution (as defined in the legend to Table I): GBM staining was described as trace or absent, normal or thickened; mesangial staining was described as trace or absent, normal, or expanded (mildly or markedly).

Autofluorescence of GBM and mesangial areas, found especially in several diabetic tissues (17), precluded satisfactory evaluation and necessitated elimination of these tissues from the results.

RESULTS

Glomerular antigens in normal renal tissue

Normal immunochemical reactions of FITC-labeled antisera on normal renal tissue are illustrated in Fig. 1 and diagramatically presented in Fig. 2 and Table II. Within the glomerulus, anti-GBM (Fig. 1A and Fig. 2) and anticollagen antisera stain the GBM almost exclusively. Antiactomyosin (Fig. 1B and Fig. 2) fixes to the mesangium.

Collagenase digestion of normal tissue, as described by Rothbard and Watson (11), abolished fluorescent staining of the GBM by anticollagen and anti-GBM, whereas antiactomyosin staining of the mesangium remained intact.

Glomerular antigens in renal disease (Table I)

Membranoproliferative glomerulonephritis. There was an almost complete loss of normal glomerular anti-

Table II
Reactivity of Antisera to Renal Structures

Location in normal kidney	Antisera		
	Anti-GBM	Anti-collagen	Antiacto-myosin
Nonglomerular			
TBM	3+	0	0
Bowman's capsule	2+	tr	0
Capillary basement membrane	2+	tr	0
Peritubular capillary wall	0	1–2+	2+
Artery walls	0	tr	1+
Interstitium	0	1–2+	tr
Glomerular			
GBM	3+	1–2+	0
Mesangium	0	tr	2+

gen localization in membranoproliferative glomerulonephritis. The expanded mesangial areas did not react with antiactomyosin, and there was only minimal staining at the periphery of the mesangial areas. The staining of the GBM by anti-GBM (Fig. 3) and anticollagen was markedly diminished or absent; even in less severely involved glomeruli, normal GBM antigens were detectable only as a fine rim around capillary loops. Certainly no splitting of the GBM was seen, as found on silver stains. One representative biopsy specimen of this disease, stained by indirect immunofluorescence with the Goodpasture's antibody, also showed trace GBM staining.

Membranous nephropathy. In membranous nephropathy, studies with anti-GBM antibody revealed thickening of the basement membranes (Fig. 4). In more severe lesions an apparent splitting of the basement membrane was seen with an accentuation of the inner layer. Occasionally, interruptions in staining of the outer aspect could be seen, probably corresponding to immune deposits. Results with anticollagen were similar, although the inner accentuation was less promi-

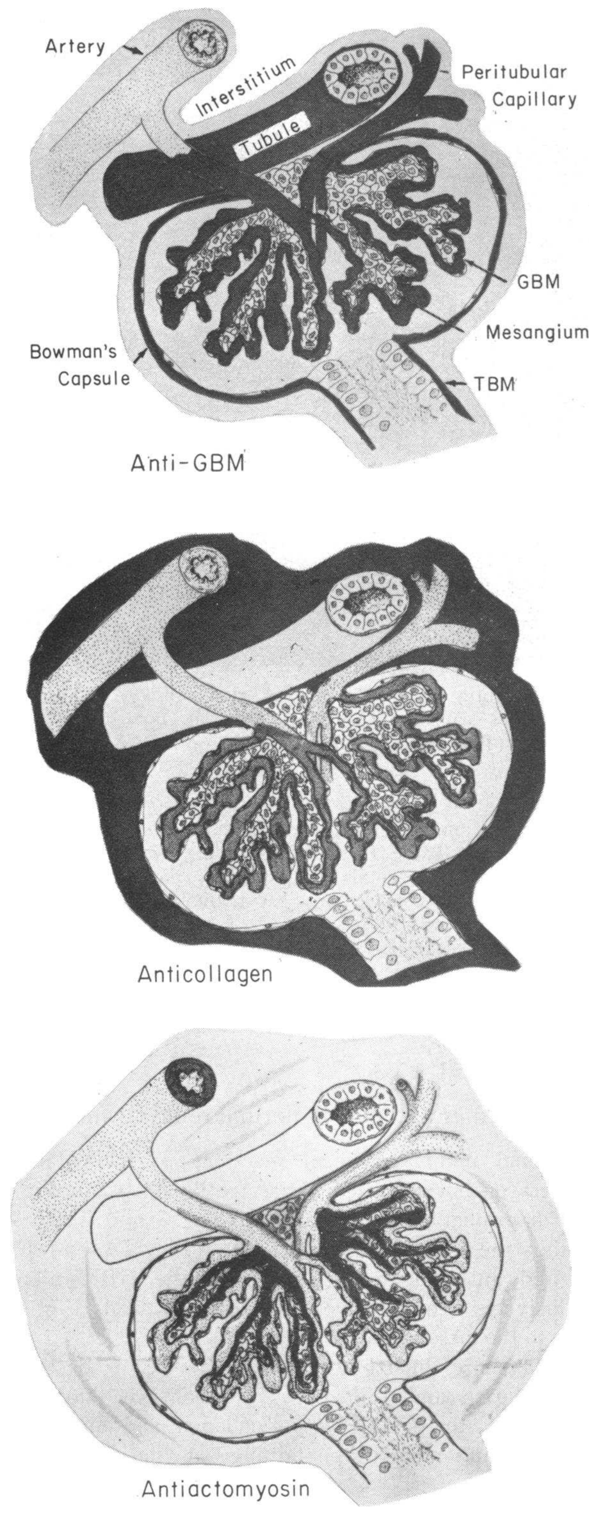

Figure 2 A composite illustration of the fluorescent staining patterns on normal kidney tissue of antisera to GBM (anti-GBM), collagen (anticollagen), and actomyosin (antiactomyosin). Gray shading indicates positive immunofluorescence. *Anti-GBM:* GBM, TBM, and Bowman's capsule are stained, along with peritubular capillary and arteriolar basement membranes. No staining of the mesangium or interstitium is found. The antibody eluted from a kidney with anti-GBM nephritis (Goodpasture's) stains only the GBM by indirect immunofluorescence. *Anticollagen:* GBM is stained, but Bowman's capsule is stained only weakly. TBM are not stained and appear as negative "lines" between the stained interstitium and the light autofluorescence of tubular cytoplasm. There is only trace staining of the mesangium. Peritubular capillary and arteriolar basement membranes are weakly stained. (Anticollagen antiserum from Dr. Michaeli stains the glomerulus diffusely and the interstitial staining is stronger). *Antiactomyosin:* GBM, TBM, and Bowman's capsule are not stained. The mesangium is prominently stained. In the interstitium most staining is associated with capillary endothelial cells or with the muscularis of arterioles and venules.

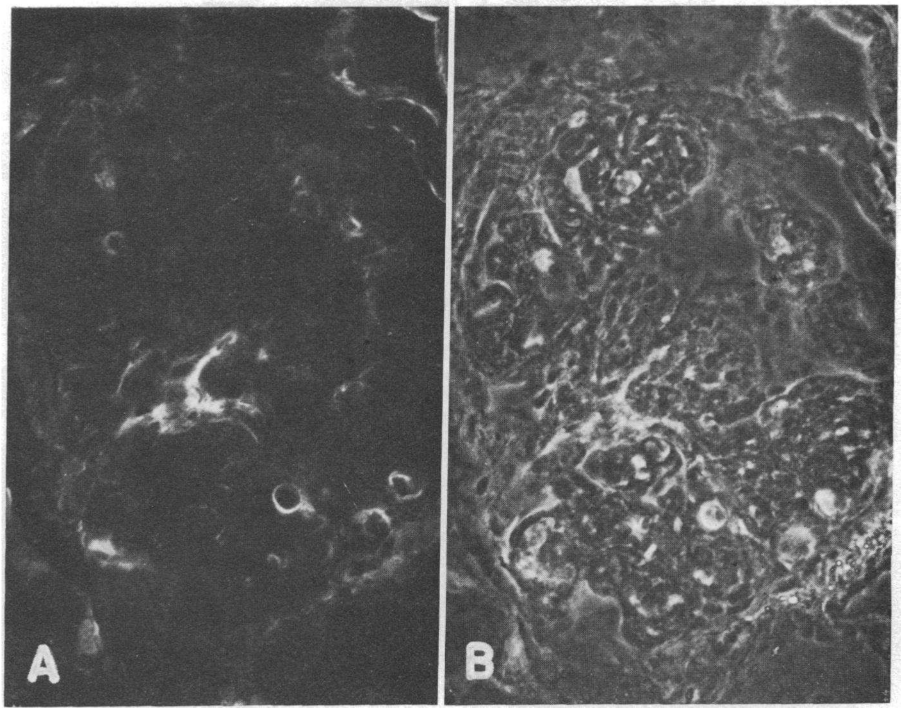

FIGURE 3 Membranoproliferative glomerulonephritis. (A) Marked mesangial expansion in glomerular lobules, viewed by indirect immunofluorescence, staining with FITC-labeled antiserum to GBM. Only trace staining of the GBM is seen. (B) Phase and indirect immunofluorescent microscopy combined, same field as A. Three circular autofluorescent artifacts are present. Magnification 400 ×.

nent. Staining of the mesangium by antiactomyosin was mildly expanded in seven of 15 patients. The one patient whose light microscopy (including silver methenanine stain) was normal (but with positive immunofluorescent staining for C3, IgM, and IgG, typical of membranous nephritis) had no immunofluorescent abnormalities with anti-GBM, anticollagen, and antiactomyosin.

Diabetes mellitus. In tissues from nine patients with renal failure due to diabetes mellitus, the distribution of actomyosin was markedly increased to encompass the full extent of the expanded mesangium seen by light microscopy (Fig. 5C [early] and 5D [late]). Absorption of the antiactomyosin with its specific antigen completely abolished this staining in the one severe case so studied. There was a widening (Fig. 5A) and apparent splitting (Fig. 5B) of the GBM with an accentuation of the inner layer when anti-GBM and anticollagen antisera were used. One of these tissues studied by indirect immunofluorescence with the Goodpasture's antibody revealed similar staining reactions of the GBM.

Five other tissues showed results generally indistinguishable from other end-stage kidneys described below. Other tissues had strong autofluorescence of

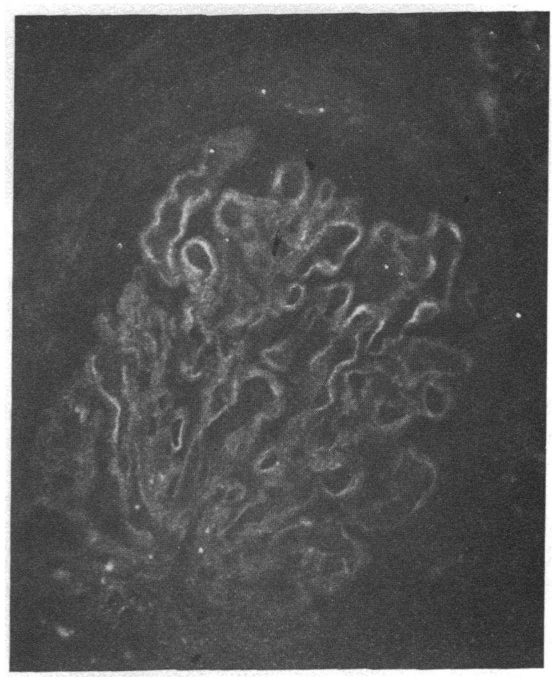

FIGURE 4 Membranous nephropathy. Immunofluorescent staining with anti-GBM shows thickened capillary loops. Magnification 600 ×.

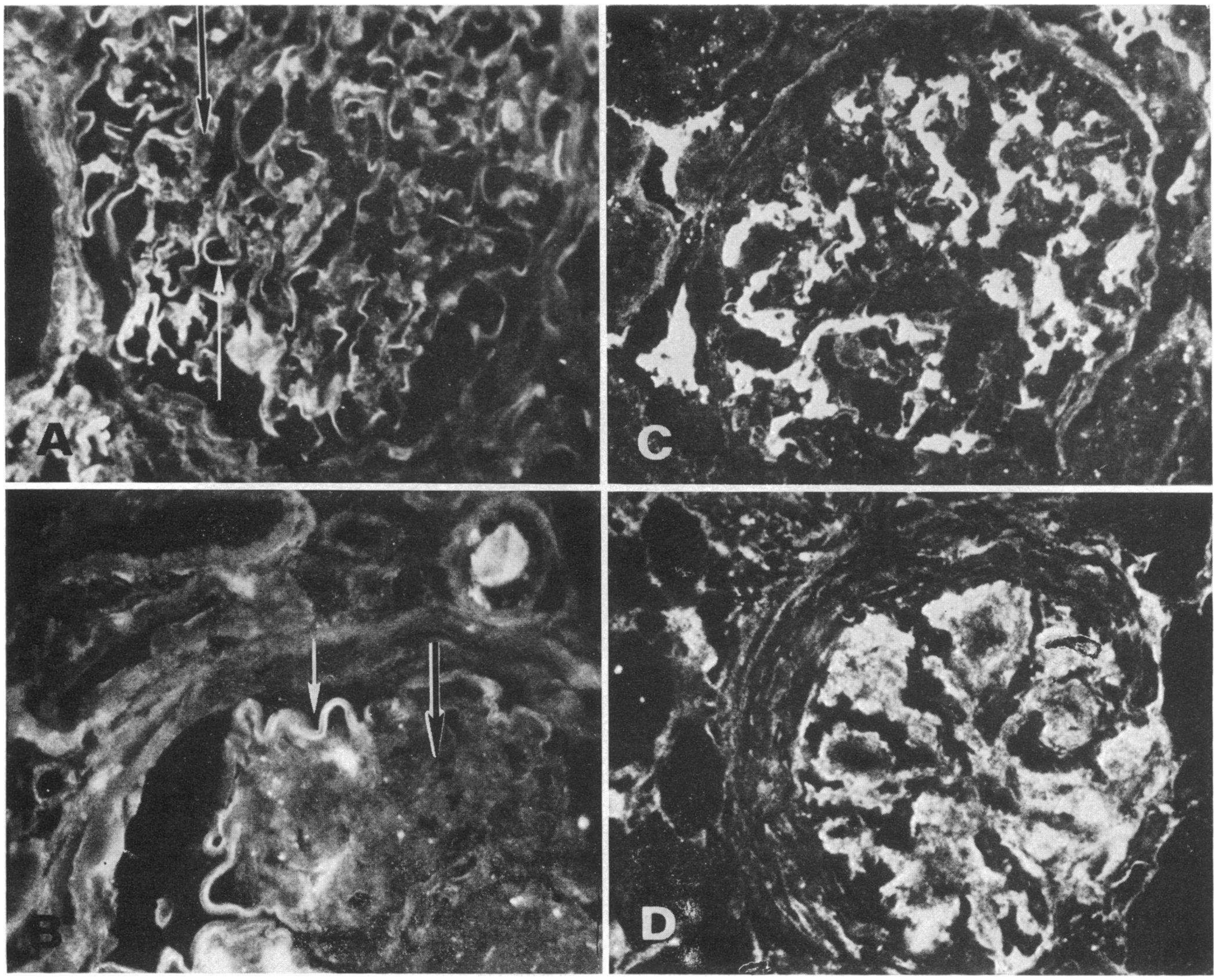

FIGURE 5 Diabetes Mellitus. Immunofluorescent staining with anti-GBM (A and B) and antiactomyosin (C and D) antisera. Anti-GBM staining in early (A) and late (B) diabetic nephropathy demonstrates thickening of basement membranes (white arrows) and unstained expanded mesangium (black arrows). Antiactomyosin staining of early (C) and late (D) diabetic nephropathy demonstrates progressive expansion of positively stained mesangium. Magnification 800 ×.

GBM (five patients) and mesangial nodular sclerotic areas (seven patients), which precluded satisfactory evaluation with our antisera.

Kidneys from three patients with early diabetes were also studied. The renal tissue of a 4-yr-old boy was studied at the onset of idiopathic nephrotic syndrome; juvenile diabetes mellitus was discovered 1 wk later and has continued for 2 yr after steroid have been discontinued. Routine light and fluorescent microscopy were normal, as were our stains for glomerular antigens. Two other patients, aged 15 and 21, with diabetes mellitus of 3 and 10 yr duration, had no evidence of basement membrane thickening or pronounced mesangial expansion by light microscopy. Electron microscopy in one showed focal areas of GBM thickening and minimal enlargement of the mesangium by electron microscopy (performed by Dr. Z. Posalaky, St. Paul-Ramsey Hospital). Anti-GBM and anticollagen antisera demonstrated focal areas of apparent basement membrane thickening in these two patients, but accentuation of the inner aspect of the GBM was not discernible. Antiactomyosin staining of the mesangium was markedly expanded.

Glomerular "hyaline" droplets, although identified by light microscopy in several of our patients with diabetic nephropathy, could not be identified with our three antisera. Intracapillary glomerular lesions characteristic of "Kimmelstiel-Wilson nodules" in mesangial areas were often strongly autofluorescent. However, patients with nodular patterns of mesangial expansion by light microscopy did not demonstrate corresponding nodular structures within the expanded mesangial areas stained with antiactomyosin.

Proliferative glomerulonephritis. Tissues of three patients with poststreptococcal and one with rapidly progressive glomerulonephritis showed an extensive distribution of staining with antiactomyosin that extended to the periphery of glomerular lobules, and often lost the mesangial pattern (Fig. 6). However, the width of the stained mesangium stalk was not increased. The glomeruli of seven patients with nephritis due to anaphylactoid purpura and focal mesangial proliferative nephritis had similar findings, although the staining of the mesangium by antiactomyosin was expanded somewhat more in width in five of these patients, corresponding to the widened mesangial areas seen by light microscopy. Anti-GBM and anticollagen staining of the GBM was normal in these 11 patients, although occasionally the continuity of the GBM could not be clearly discerned.

Fibroepithelial crescents in one patient with rapidly progressive nephritis, in two of the children with anaphylactoid purpura, and in one with mesangial proliferative nephritis were minimally stained with our antisera; there was a weak reticular staining, apparently surrounding cells, seen with anticollagen and, still weaker, with antiactomyosin. Focal sclerotic areas of glomeruli in a patient with mesangial proliferative nephritis were not stained with any of our antisera.

Miscellaneous renal disease. Patients with nephrotic syndrome had no clear abnormalities by immunofluorescent staining for glomerular antigens except focal areas of decreased GBM staining with anti-GBM in three patients. Focal sclerotic areas of glomeruli were not stained by any of the three antisera in the six patients with segmental hyalinosis.

The hypertrophied glomeruli of a child with cyanotic congenital heart disease were stained more extensively than normal with all three antisera, rendering clear demarcation of GBM and mesangial zones impossible. The end-stage kidneys of the patient with renal hypoplasia and oligonephronia had normal glomerular antigens.

The expanded mesangium in amyloid nephropathy, positively stained by thioflavine T, was not stained with antiactomyosin which stained only narrow areas of the mesangium surrounding the areas of amyloid deposition.

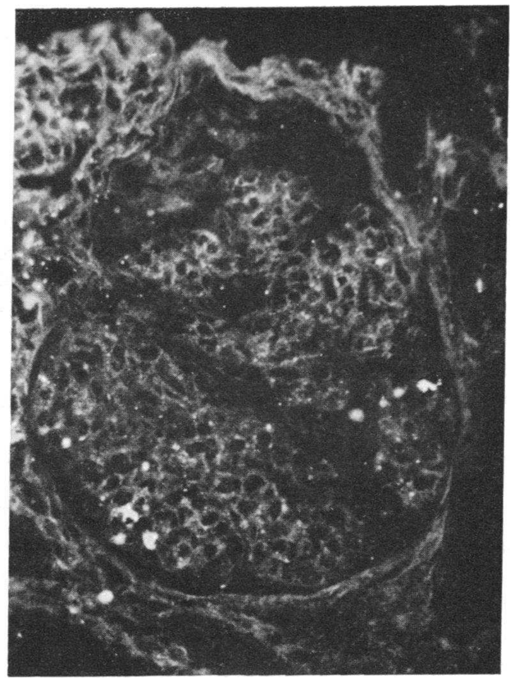

FIGURE 6. Acute poststreptococcal glomerulonephritis. Immunofluorescent staining with antiactomyosin is more extensive than normal, though of lower intensity than that seen in diabetes mellitus. The staining is present throughout the glomerulus; its precise location is unknown. Increased staining in the interstitium is visible, without staining of TBM. Magnification 700 ×.

End-stage kidney. Nephrectomy specimens of 12 end-stage kidneys showed large numbers of completely hyalinized glomeruli, which failed to react with any of our three antisera. Anticollagen showed an extensive staining reaction in the interstitium, while hyalinized glomeruli were negative. The anticollagen antiserum supplied by Dr. Michaeli showed the same findings in four specimens examined by indirect immunofluorescence. The antiactomyosin antiserum showed some increased reactivity in the expanded interstitium while anti-GBM remained negative. In the interstitium of the child with renal hypoplasia with oligonephronia, a greatly increased distribution and intensity of staining by antiactomyosin was noted, even more than that to anticollagen antiserum.

DISCUSSION

In renal diseases with distinctive histopathologic characteristics there are alterations in antigenic components which react with antibody to human GBM, acid-soluble skin collagen, and uterine smooth muscle actomyosin. Within the normal glomerulus, anti-GBM and anticollagen antibodies react with GBM whereas antiactomyosin localizes to the mesangium.

The most striking increase in actomyosin was observed in the nephropathy of diabetes mellitus where the distribution and quantity was correlated with expansion of the mesangium characteristic of diabetic nephropathy. In contrast, the widened mesangial matrix observed in membranoproliferative glomerulonephritis did not contain identifiable actomyosin or collagen antigens. Although large amounts of actomyosin in the sclerotic mesangium of diabetic glomeruli could reflect the presence of residual cellular debris, this finding was not observed in other diseases with glomerular sclerosis.

It is thus possible that the increased actomyosin-like material found in the mesangium in diabetes mellitus does not represent a functioning contractile system, but instead a specific defect in the normal mechanisms for removal of cellular debris from the mesangium; preliminary data suggests a defective clearance of exogenously administered aggregated gamma globulin from the mesangium in alloxan diabetic rats (18). If the increased actomyosin-like protein found in the mesangium in diabetes mellitus represents a primary phenomenon, its role in the pathogenesis of diabetic nephropathy may be related to diabetic vascular disease elsewhere. The early development of atherosclerosis in diabetes mellitus may be related to proliferation or alteration in smooth muscle proteins. While hypertension may stimulate vascular smooth muscle proliferation (19), our two early cases of diabetes mellitus had no evidence of hypertension, but definite increase in mesangial actomyosin. Defects in leukocyte function in diabetes mellitus (20) may also be related to alterations in smooth muscle proteins. Contractile protein is prominent in leukocytes (21) and apparently responsible for their motility (22) which is altered in the diabetic state (20).

In other disease states, such as proliferative glomerulonephritis and many cases of membranous nephropathy, actomyosin is mildly increased in distribution by immunofluorescence; while this may be related to expansion of mesangial components (23), the appearance of actomyosin in epithelial cells is equally likely and would correspond to the increase in smooth muscle-like structures in epithelial cells seen by De Martino, Accinni, and Procicchiani (24) in proliferative glomerulonephritis. However, the immunofluorescent alterations seen in disease states may not represent the same determinants of actomyosin responsible for the normal mesangial staining.

The presence of actomyosin antigen(s) in the glomerular mesangium suggests but does not prove that functioning contractile elements are present in this locus. Ultrastructural localization of thin filaments (24), presumably actin (25), in the glomerulus does not show the mesangial preponderance that we demonstrated by immunofluorescence. Smooth muscle myosin, considered by some (3) to be the predominant antigenic determinant of actomyosin, is poorly demonstrated by electron microscopy.

The presence of contractile proteins in the mesangium may play an important role in regulating glomerular blood flow and ultrafiltration (26). This is particularly relevant because of the contiguity of the mesangium with smooth muscle cells in afferent arterioles and the juxtaglomerular apparatus (27).

The absence of significant glomerular fluorescence with antisera to GBM and collagen in membranoproliferative glomerulonephritis suggests that these antigens may be altered or lost. The basement membrane-like material seen by electron microscopy in the mesangium in membranoproliferative glomerulonephritis may be (antigenically) abnormal GBM, and its synthesis by mesangial cells may be related to the pathogenesis of the disease. The apparent splitting of the GBM observed by silver methenamine stain in membranoproliferative glomerulonephritis was not seen in our studies with anti-GBM and anticollagen sera. That splitting seen with silver methenamine stain may be related to components of the glomerular capillary wall other than GBM. In contrast, however, a laminated structure of the thickened GBM was seen in diabetes mellitus and membranous nephropathy, with the inner layer more intensely stained by anti-GBM antiserum; it was also found with the eluted Goodpasture's anti-GBM antibody. The relationship between this finding and the ultrastructurally observed laminae rara and densa of the GBM is unknown.

Localization of heterologous anti-GBM serum to glomerular and tubular basement membranes, Bowman's capsule, and capillary basement membranes is well established. The more restricted pattern of staining seen with Goodpasture's antibody, staining mainly GBM (1), suggests a more specific reactivity with GBM antigens. Anticollagen antibody localized to GBM as shown by Rothbard and Watson (2), but is clearly differentiated from anti-GBM antibody by staining of the interstitium and virtual absence of TBM staining. Anti-GBM and anticollagen antibody staining of the GBM were abolished by collagenase digestion of tissues before staining. These data suggest that our anti-GBM and anticollagen antibodies both may react with collagen-related proteins. However, the results of absorption and tissue fixation studies clearly differentiate these two antisera. Previous studies have emphasized the numberous antibodies elicited by collagen (14), GBM (28), and other basement membranes (29). The immunochemical relationship between different collagenlike proteins and fractions of their molecules awaits further clarification.

Our studies confirm the presence of large amounts of collagen in renal tissue with interstitial fibrosis. However, we could not demonstrate collagen in hyalinized glomeruli with our antiserum, nor with that of Dr. Michaeli. Similarly, we found almost no localization of anticollagen or anti-GBM antibody to fibrosing locules of glomeruli with focal sclerosis, little in fibroepithelial crescents, and none in the expanded mesangial areas of diabetic nephropathy and membranoproliferative glomerulonephritis. These areas may contain collagenous material, but its physiochemical state may have been altered and antigenic determinants not available for immunofluorescent reactions. Although the cells of fibroepithelial crescents have been considered "myofibroblasts" (30), we found no evidence for the presence of actomyosin. Although interstitial localization of actomyosin is minimal in the normal kidney, it is clearly present in specimens from patients with renal insufficiency and interstitial fibrosis. A similar observation was made by Nagle, Kneiser, Bulger, and Benditt (31) in obstructive uropathy in the rabbit using a human smooth muscle autoantibody (32).

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