0023-6837/83/4901-0107$02.00/0
LABORATORY INVESTIGATION

Vol. 49, No. 1, p. 107, 1983
Printed in U.S.A.

Methods in Laboratory Investigation

Monoclonal Antibodies against Rat Glomerular Antigens: Production and Specificity

DONNA L. MENDRICK, HELMUT G. RENNKE, RAMZI S. COTRAN, TIMOTHY A. SPRINGER, AND ABUL K. ABBAS

Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts

To define the characteristics and target antigens (Ags) of nephrotoxic antibodies (Abs) and to analyze the factors that govern the evolution of Ab-mediated glomerular injury, we have prepared monoclonal Abs against rat glomerular Ags. BALB/c mice were immunized with Lewis rat cortex or glomeruli, and their spleens were removed and fused with hypoxanthine-aminopterin-thymidine supplement-sensitive myelomas. Hybrids were selected for production of Abs against Lewis rat kidney by indirect immunofluorescence. To date, more than 50 positive hybrids have been selected and their tissue reactivity defined by indirect immunofluorescence and immunoelectron microscopy. Of these, 14 are presented here in detail. One of these monoclonal Abs, K9/4, recognizes a unique Ag present exclusively on the cell surface of rat glomerular visceral epithelial cells. Four Abs (K12/2, K17/4, K12/5, and K12/8) recognize sites within the glomerular basement membrane; K12/2 and K17/4 also bind to vascular basement membranes of the rat, whereas K12/5 and K12/8 bind to glomerular basement membrane, tubular basement membranes, and vascular and epithelial basement membranes in all tissues of the rat. Two hybridomas (K6/1 and K6/3) recognize determinant(s) present on cell surfaces of endothelial and epithelial cells as well as within the glomerular basement membrane. All of these previously mentioned Abs are species restricted (*i.e.*, they bind only to rat tissue) and, with the exception of K9/4, bind upon *in vivo* administration. Several others, however, recognize ubiquitous Ags that are present on intracellular structures in every species tested. The tissue distribution of these Ags suggests that they are present in contractile or cytoskeletal elements and, as expected from their intracellular location, monoclonal Abs directed against these components do not bind upon *in vivo* administration. Future studies will be directed at defining the antigenic composition of the glomerular capillary wall and the relevance of such Ags in immune-mediated glomerular injury.

Additional key words: Glomerular basement membrane, Immunoelectron microscopy.

The roles of immune complexes and antikidney antibodies in the pathogenesis of glomerular disease have been recognized for more than two decades. However, progress in this area has been hampered by two problems. First, the nature and localization of the antigens (Ags) that are the targets for nephritogenic antibodies (Abs) are poorly defined. Recent studies have demonstrated the presence of collagen (types IV and V), laminin, glycosaminoglycans, entactin, and possibly fibronectin in the glomerular basement membrane (GBM) (4, 6, 7, 9, 15, 21, 25, 26, 29, 30). The nephrotoxicity of purified Abs directed against the collagenous constituents of the glomerular capillary wall remains controversial (8, 16, 34), although most studies suggest that the major nephrotoxic Abs of the GBM are noncollagenous glycoproteins (8, 11, 16, 22, 28). Second, experimental glomerular injury can be induced by a variety of mechanisms that may or may not be related to complement activation and inflammatory cell influx. It is unclear whether such differences reflect variations in the Abs used to produce disease, the nature of the target Ags or the experimental models.

In an effort to study the pathogenesis of experimental glomerulonephritis in a more precise and quantitative manner, we have attempted to produce monoclonal Abs specific for antigenic determinants of the rat kidney. The feasibility of producing functional and morphologic disease with monoclonal Abs has already been demonstrated in experimental myasthenia gravis (14, 20) and myeloma neuropathy (5). Such Abs can also serve as affinity probes for Ag localization and purification, an approach that could provide valuable information about the composition of the glomerular capillary wall and the biosynthesis of its constituents. In this paper we describe the characteristics and binding patterns of monoclonal Abs that react with various components of the rat glomerular

capillary wall. These studies demonstrate the presence of some antigenic determinants that are unique to rat glomerular structures and others that are shared among different renal and nonrenal tissues.

MATERIALS AND METHODS

PRODUCTION OF MONOCLONAL ABS

Immunization. BALB/c mice (Cumberland View Farms, Clinton, Tennessee) were immunized with either 0.01 ml of 20% v/v homogenized kidney cortex or 0.01 mg of isolated glomeruli obtained from Lewis rats (125 to 150 gm) (Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts). Glomeruli were isolated by the sieve technique from kidneys perfused in situ with cold (4° C) phosphate-buffered saline solution (PBS, 0.145 M NaCl, 0.01 M phosphate buffer, pH 7.4). The antigenic preparations were emulsified in complete Freund's adjuvant containing 5 mg/ml of H37 RA *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, Michigan) and injected into the footpads. Mice were boosted every 2 weeks with either glomeruli or homogenized kidney cortex in incomplete Freund's adjuvant. Mice were tested for serum Abs to rat kidney by indirect immunofluorescence (IIF). Three days prior to fusion, a selected mouse was given an intraperitoneal injection of renal cortex or glomeruli in saline.

Hybridization. Spleen cells of immunized mice were fused with either a P3-NS1/1-Ag4-1 (NS1) or the FO varient of P3-X63-Ag8 using 50% (w/w) polyethylene glycol 1500 (BDH Chemicals Ltd., Poole, England) at a 4:1 ratio of splenocytes to myeloma cells. The cells from such a fusion were plated in Costar 96-well plates (M.A. Bioproducts, Bethesda, Maryland) and cultured for 2 weeks in RPMI 1640 containing 1 mM L-glutamine, 1 mM sodium pyrivate, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (M.A. Bioproducts), 20% decomplemented fetal calf serum (Gibco Laboratories, Grand Island, New York), and hypoxanthine-aminopterin-thymidine supplement (final concentrations in culture medium 100, 0.4, and 16 μM, respectively) (Sigma Chemical Company, St. Louis, Missouri). During these 2 weeks the cultures were fed on days 7 and 10. The culture medium was changed on day 14 to RPMI 1640 containing L-glutamine, sodium pyruvate, Hepes buffer, 20% decomplemented horse serum (M.A. Bioproducts), hypoxanthine, and thymidine, with feedings on day 14, 16, and 18. On day 21, hypoxanthine and thymidine were omitted and the cultures were fed every 2 days.

Screening for Production of Kidney-Binding Antibody. Supernatants of growing cultures were tested for the production of kidney-fixing Abs by IIF microscopy. Three-micrometer thick frozen sections of Lewis rat kidney were incubated with 50 μl of culture supernatants in a humid chamber at room temperature for 30 minutes, washed thrice with PBS, and incubated with fluorescein isothiocyanate-conjugated (FITC) rabbit antimouse immunoglobulin (RAMG) for 30 minutes. This Ab was obtained from rabbits immunized three to five times with mouse immunoglobulins in complete Freund's adjuvant; an IgG fraction was purified from this serum by 45% ammonium sulfate precipitation and ion exchange chromatography. After incubation with FITC-RAMG, sections were washed three times in PBS, mounted in PBS glycerin mixture (1:9), and examined with a Leitz Orthoplan fluorescence microscope equipped with a Ploemopak 2.1 epiiluminater.

Cloning. Hybrids were cloned in soft agar, supernatants were tested for antikidney activity, and positive clones were subcloned once. Cloned cultures were grown and maintained in RPMI 1640 with L-glutamine, sodium pyruvate, Hepes buffer, and 10% decomplemented horse serum. Hybridoma-derived Abs for *in vivo* studies were produced in BALB/c mice in ascites form. For this purpose, cloned hybrids (2×10^6 cells/mouse) were injected into the peritoneal cavities of mice that had received 1 ml of 2,6,10,14,tetramethylpentadecane (96%, Aldrich Chemical Company, Milwaukee, Wisconsin) 7 to 30 days previously, the ascitic fluid was collected in heparinized tubes, and globulins were isolated by 45% ammonium sulfate precipitation. After dialysis in PBS, aliquots were frozen and kept at −20° C.

TEST FOR TISSUE AND SPECIES SPECIFICITY

To determine whether the monoclonal Abs were directed against rat-specific determinants, Lewis rat thymocytes were incubated for 30 minutes at 4° C with culture supernatants or sera obtained from mice immunized with rat kidney Ags (positive control), washed, and stained with FITC-RAMG for 30 minutes at 4° C. Cells were washed twice, fixed in 1% paraformaldehyde for 15 minutes, and examined by fluorescence microscopy as described before. The reactivity of hybridoma-derived Abs with other rat tissues (heart, lung, aorta, liver, choroid plexus, adrenal, spleen, intestine, and esophagus) and with kidneys obtained from mice, guinea pigs, rabbits, and humans was tested by IIF on frozen sections as described before.

IMMUNOCHEMICAL PROPERTIES OF MONOCLONAL ABS

Class and subclass of monoclonal Abs were determined on culture supernatants by double diffusion in agar using rabbit antisera specific for different mouse immunoglobulin heavy and light chains (Miles Laboratories, Inc., Elkhart, Indiana). The isoelectric points (pIs) of hybridoma-derived Abs were determined by slab-isoelectric focusing in acrylamide gels containing 4.5 gm/100 ml of acrylamide/bis/acrylamide (Bio-Rad Laboratories, Richmond, California), 4 gm/100 ml of glycerol. Polymerization was achieved on silane-coated class plates by ammonium sulfate and TEMED (Bio-Rad). Radioactive labeling of Abs was accomplished by ^{35}S-methionine incorporation. For this purpose, 1.5×10^6 hybrid cells were placed in 1 ml of methionine-free RPMI 1640 (ISI Biologicals, Gary, Indiana) with L-glutamine, sodium pyruvate, Hepes buffer, and 10% decomplemented fetal calf serum and supplemented with 20% (v/v) methionine-containing culture medium and 50 μCi of ^{35}S-methionine (New England Nuclear, Boston, Massachusetts). Supernatants were harvested after 18 to 24 hours,

dialyzed against distilled water for 48 hours, aliquoted, and frozen. Samples containing 10^4 cpm of trichloroacetic acid-precipitable ^{35}S-labeled protein were loaded on preformed wells in the acrylamide gel. Electrofocusing was performed at a constant current/constant voltage of 1 ma/band and 300 volts for 4.5 hours at 4° C. At the end of the electrophoretic procedure the pH on the gel was measured at 0.5-cm intervals by means of a surface microelectrode (Ingold Electrodes, Andover, Massachusetts) and Radiometer PM 62 pH meter (The London Company, Cleveland, Ohio). The gel was stained on the glass with Coomassie blue R250 (Bio-Rad), destained in methanol-acetic acid-water (4:1:5) mixture, and dried at room temperature overnight. Radioautography was performed by exposing Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, New York) for 3 to 7 days.

Immunoelectron Microscopy

Sieve-isolated glomeruli were slightly disrupted by sonication (3 to 5 seconds) at 4° C with a W 220F sonicator (Heat Systems-Ultrasonics, Inc., Plainview, New York) to yield glomerular fragments devoid of Bowman's capsule, thereby facilitating diffusion and binding of Abs to the different elements of the glomerular capillary wall. The glomeruli were kept at 4° C until fixation. After 2 washes with PBS containing 1% normal rabbit serum, glomeruli derived from one kidney were incubated with a monoclonal Ab or a dilution of mouse serum for 30 to 60 minutes. (To assure uniform immunoglobulin concentrations in the culture supernatants of the monoclonal Abs, the amount of immunoglobulin was quantitated by single radial immunodiffusion (Mancini)). After three washes with PBS with 1% rabbit serum, glomeruli were incubated with a predetermined optimal dilution of horseradish peroxidase-labeled RAMG or horseradish peroxidase-Fab RAMG for 30 to 60 minutes prepared as described by Nakane and Kawaoi (17). After three washes with 0.2 M cacodylate buffer (pH 7.4), the glomeruli were fixed in half-strength paraformaldehyde-glutaraldehyde mixture (pH 7.4) (13) for 30 minutes and then washed three times with 50 mM tris-(hydroxymethyl)aminomethane-HCl buffer (pH 7.6). Peroxidase histochemistry was performed as described by Graham and Karnovsky (10). Glomeruli were kept in the diaminobenzidine solution for 15 minutes, washed two times with 0.2 M cacodylate buffer, postfixed in OsO_4 (1 gm/100 ml) for 15 to 30 minutes, dehydrated in graded ethanols, and embedded in epoxy resin. Unstained 1-μm thick sections were cut with glass knives and examined by light microscopy. Thin sections were cut with diamond knives on a LKB Ultratome III and viewed unstained in a Philips 201 electron microscope at 60 kv.

In Vivo Localization of Monoclonal Abs

Monoclonal Abs produced as ascitic fluid were injected into the tail vein of unanesthetized Lewis rats. The Abs were injected in a solution containing 20 to 40 mg of total globulin. Three days after the injection, kidneys were removed and 3-μm frozen sections were stained with FITC-RAMG as described previously (direct immunofluorescence microscopy).

Controls

The specificity of binding patterns by IIF and immunoelectron microscopy was tested by using nonkidney-binding monoclonal Abs of various classes and subclasses and preimmune mouse serum as negative controls; sera obtained from mice immunized with rat kidney Ags served as a positive control. To determine the specificity of *in vivo* localization of monoclonal Abs, rats were injected with equivalent amounts of various nonkidney-fixing monoclonal Abs, preimmune and immune mouse serum globulins, and binding to kidneys was assessed by fluorescence microscopy.

RESULTS

To date more than 50 monoclonal Abs reactive with Lewis rat kidneys have been isolated; 14 of these recognize components of glomerular capillary walls, and their binding and immunochemical properties are the subject of this report. Analysis of binding patterns to renal and nonrenal tissues indicates that these Abs react with Ags that can be divided into four broad categories. (a) One monoclonal Ab recognizes an antigenic determinant present exclusively on visceral epithelial cells of the kidney. (b) Two monoclonal Abs recognize antigenic determinants on the surfaces of rat glomerular epithelial and endothelial cells as well as within the GBM. (c) Four other Abs are specific for constituents of extracellular basement membranes in the rat, and their reactivity is not restricted to glomeruli alone. (d) Seven Abs bind to intracytoplasmic Ags in cells in glomeruli and a variety of nonrenal tissues; only two of the fourth group are species specific, and the pattern of distribution of the relevant Ags suggests that they are components of contractile or cytoskeletal systems. All of these Abs fail to bind to viable Lewis rat thymocytes in suspension, indicating that they are not directed against a common rat-specific determinant(s) such as major histocompatibility Ags.

Abs Reactive with Epithelial Cell Surfaces

K9/4. K9/4 is an IgG_1, K Ab whose major band has a pI of 8.13 and exhibits a highly restricted specificity. By fluorescence microscopy it binds diffusely to the glomerular capillary wall in a coarse pattern (Fig. 1*a*). Immunoelectron microscopy reveals that this Ab binds to a cell surface Ag(s) of all glomerular visceral epithelial cells (podocytes) (Fig. 1*b* and *d*). This Ag is not present in any other cells of the rat or in glomeruli of other species (Tables 1 and 2). Moreover, the Ab does not bind to glomeruli after intravenous administration as expected from its specificity for the epithelial cell which is inaccessible to circulating macromolecules under normal conditions of filtration.

Abs Reactive with Glomerular Cell Surfaces and Extracellular Matrices

K6/1, K6/3. These two Abs bind to the surfaces of glomerular epithelial and endothelial cells and to the matrix of the GBM (Fig. 2). They also bind to peritubular capillaries and endothelial cells of nonrenal vessels but

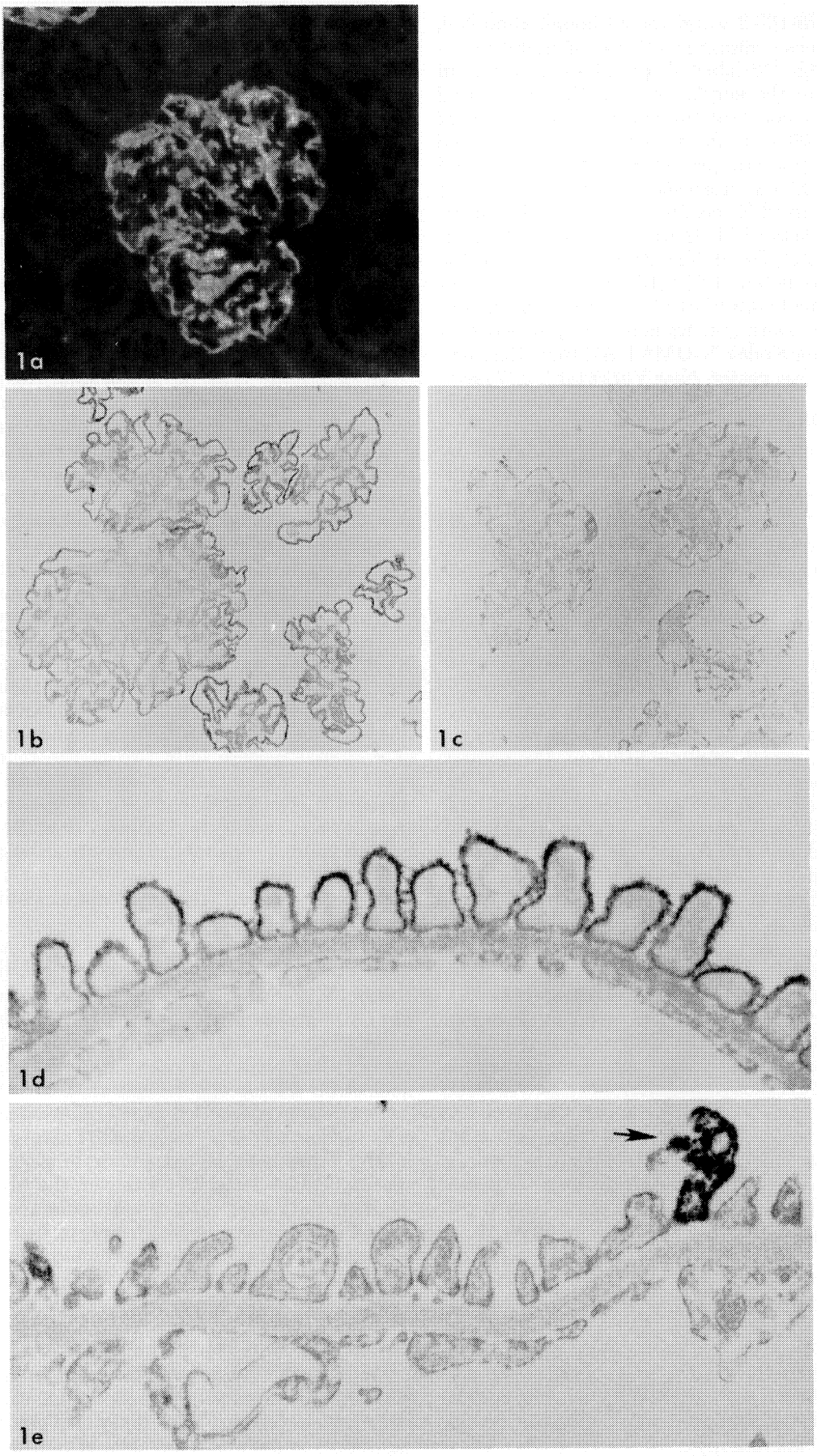


FIG. 1

TABLE 1. BINDING PATTERNS OF MONOCLONAL ANTIBODIES TO RAT KIDNEY BY INDIRECT IMMUNOFLUORESCENCE, IMMUNOELECTRON MICROSCOPY, AND AFTER *IN VIVO* ADMINISTRATION[a]

Ab	Class	IIF				IEM				*In vivo*	Rat specific
		GCW	PTC	TBM	BB	EP	END	GBM	CYT		
K9/4	$IgG_1\kappa$	C	−	−	−	+	−	−	−	−	Yes
K6/1	$IgG_{2a}\kappa$	C	+	−	−	+	+	+	−	+	Yes
K6/3	$IgG_1\kappa$	C	+	−	−	NE	NE	NE	NE	+	Yes
K12/5	$IgG_1\kappa$	L	+	+	−	−	−	+	−	+	Yes
K12/8	$IgG_1\kappa$	L	+	+	−	−	−	+	−	+	Yes
K12/2	$IgG_1\kappa$	L	+	−	−	−	−	+	−	+	Yes
K17/4	$IgG_1\kappa$	L	+	−	−	−	−	+	−	+	Yes
K8/8	IgM	I	+	−	+	−	−	−	+	−	No
K9/3	IgM	I	+	−	−	−	−	−	+	−	No
K9/7	IgM	I	−	−	−	−	−	−	+	−	Yes
K17/2	IgM	I	+	−	+	−	−	−	+	NE	No
K8/3	IgM	I	−	−	−	−	−	−	+	−	No
K17/6	IgM	I	+	−	−	−	−	−	+	−	Yes
K17/7	IgM	I	−	−	+	−	−	−	+	NE	No

[a] Abbreviations used are IEM, immunoelectron microscopy; GCW, glomerular capillary wall; PTC, peritubular capillary; TBM, tubular basement membrane; BB, brush border of proximal convoluted tubule; EP, glomerular epithelial cell surface; END, endothelial cell surface; CYT, cytoplasmic elements in glomerular cells; C, coarse, diffuse staining of GCW; L, fine linear staining; I, irregular, interrupted staining along the GCW; NE, not examined. +, positive binding; −, negative binding.

TABLE 2. TISSUE SPECIFICITY OF MONOCLONAL ANTIBODIES TESTED BY INDIRECT IMMUNOFLUORESCENCE IN THE RAT[a]

	SmMC	CaMC	StMC	Chond	Epith	End	SpVen	VasBM	EpBM	SmMM	StMM
K9/4	−	−	−	−	−	−	−	−	−	−	−
K6/1	−	−	−	−	−	+	−	?	−	−	−
K6/3	−	−	−	−	−	+	−	?	−	−	−
K12/5	−	−	−	−	−	−	−	+	+	+	+
K12/8	−	−	−	−	−	−	−	+	+	+	−
K12/2	−	−	−	−	−	−	−	+	−	+	−
K17/4	−	−	−	−	−	−	−	+	−	+	−
K8/8	d	Z	Z	+	a	+	+	−	−	−	−
K9/3	d	Z	Z	−	a, b	−	+	−	−	−	−
K9/7	−	d	d	+	c	+f	−	−	−	−	−
K17/2	d	d	d	+	b	+	+	−	−	−	−
K8/3	−	−	−	+	−	+	+	−	−	−	−
K17/6	−	−	−	−	−	+	+	−	−	−	−
K17/7	−	−	−	−	e	−	+	−	−	−	−

[a] Abbreviations used are SmMC, smooth muscle cells (media of arteries, muscle cells in intestinal lamina propria, muscularis mucosae, muscularis externa, bronchial, and esophageal smooth muscle cells); CaMC, cardiac muscle cells; StMC, striated muscle cells (psoas muscle, esophagus); Chond, chonchrocytes (trachea, bronchi); Epith, epithelium; End, endothelial cells; SpVen, splenic venules (or venous sinuses); VasBM, vascular basement membrane (capillaries and larger vessels in all organs); EpBM, epithelial basement membrane (basement membrane of renal tubules, intestinal mucosa and crypts, esophageal and bronchial mucosa, choroid plexus, hepatic ducts, and alveolar septae); SmMM, smooth muscle matrix; StMM, striated muscle matrix; d, diffuse cytoplasmic staining; Z, Z lines; a, basal layer of esophageal and tracheal epithelium; b, brush border or terminal web of intestinal bronchial and tracheal epithelium; c, brush border or terminal web of intestinal mucosa and liver ductules, diffuse cytoplasmic staining of tracheal and bronchial epithelium; e, brush border or terminal web of tracheal epithelium; f, endothelium of large vessels only, capillaries are consistently negative. +, positive binding; −, negative binding.

are specific for the rat (Tables 1 and 2). After intravenous administration, both Abs bind diffusely to glomerular capillary walls in a coarse, irregular pattern (Fig. 2*b*). The pIs of the major bands of K6/1 range from 8.00 to 8.30.

ABS REACTIVE WITH MATRICES OF BASEMENT MEMBRANES

K12/5 (pI 8.19 to 8.58), K12/8 (pI 5.57), K12/2 (pI 6.83 to 8.58), and K17/4 (pI 8.06 to 8.41) are four ex-

FIG. 1. Binding pattern of monoclonal Ab K9/4 to glomerular structures. *a*, Indirect immunofluorescence. This Ab binds to the glomerular capillary wall in a coarse, diffuse pattern. Note absence of binding to peritubular capillaries and basement membranes. *b*, By immunoperoxidase technique, diaminobenzidine reaction product is present on the peripheral capillary loops of all glomeruli. *c*, Isolated glomeruli exposed to a nonreactive monoclonal Ab of the same class and subclass as K9/4. Note absence of specific reaction product. Small areas of nonspecific staining can be observed on individual foot processes, representing elements damaged during the isolation and disruption procedure. *d*, Immunoelectron microscopy reveals reaction product exclusively on cell surface of epithelial foot processes. Endothelial cell surfaces and the GBM do not bind this Ab. *e*, Immunoelectron microscopy of a nonreactive monoclonal Ab. Note diffuse cytoplasmic staining of damaged foot process (*arrow*). Figure 1a, ×366; *b* and *c*, ×195; *d* and *e*, ×32,000.

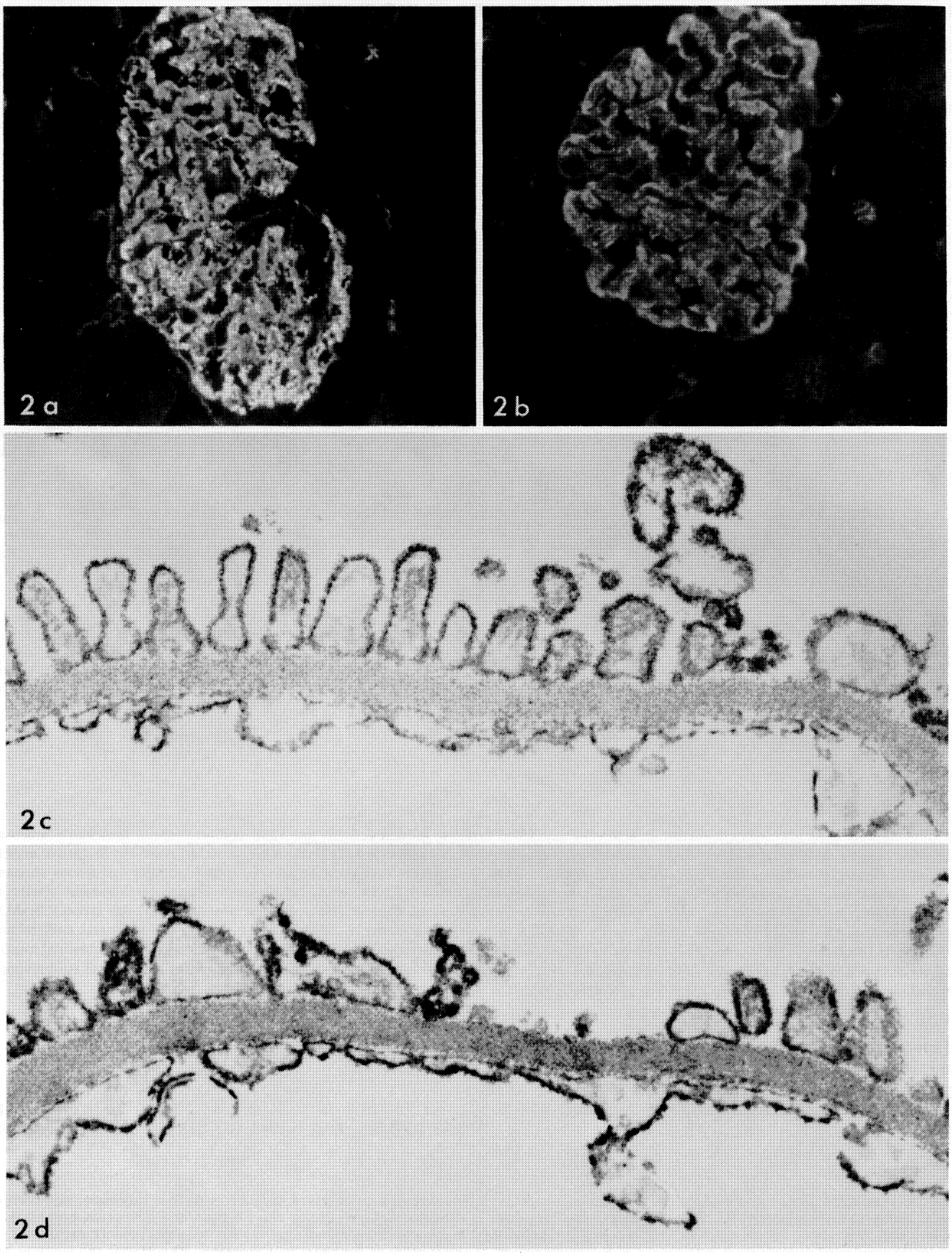
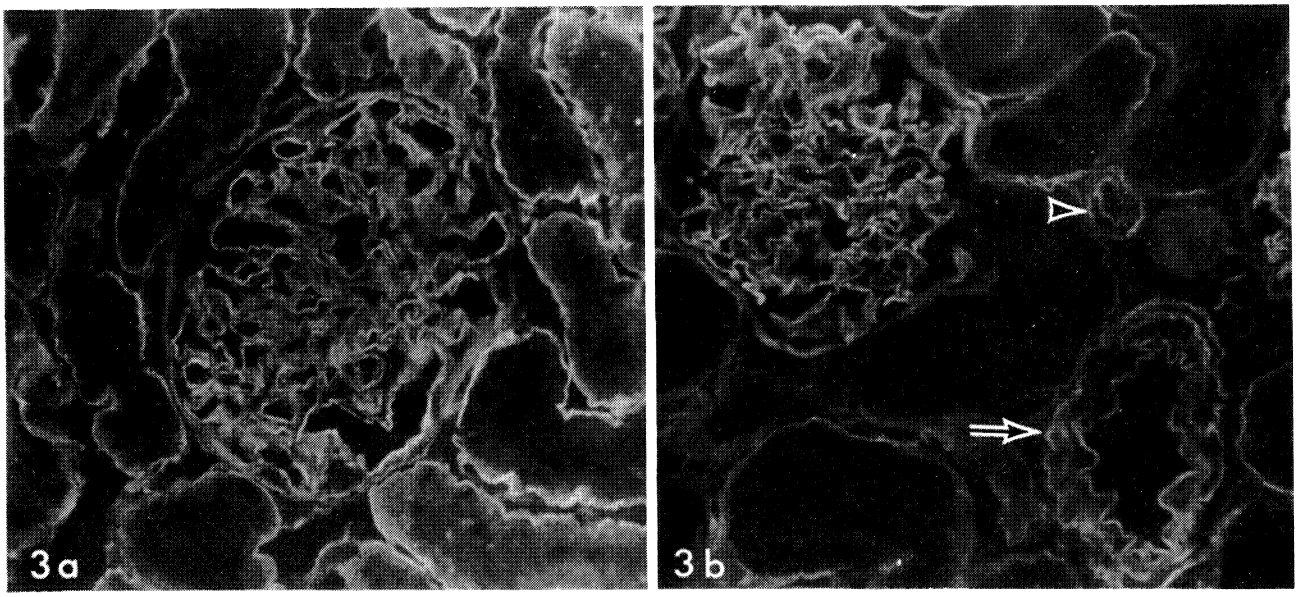


FIG. 2. Binding pattern of monoclonal Ab K6/1 to the glomerular capillary wall. *a*, IIF shows diffuse coarse staining of the glomerular capillary wall and along the endothelial lining of peritubular capillaries. *b*, Distribution of K6/1 upon *in vivo* administration of this Ab to Lewis rats. There is a thick, irregular, ribbon-like binding along the glomerular capillary wall and fine linear staining of peritubular capillaries. *c*, Immunoelectron microscopy reveals diffuse reaction product along endothelial and epithelial cell surfaces and within the GBM. Amount of reaction product is markedly increased in areas of the basement membrane devoid of epithelial cells (*d*), presumably due to facilitated diffusion and binding of the Ab to antigenic sites present in this location. Figure 2*a* and *b*, ×481; *c* and *d*, ×42,000.



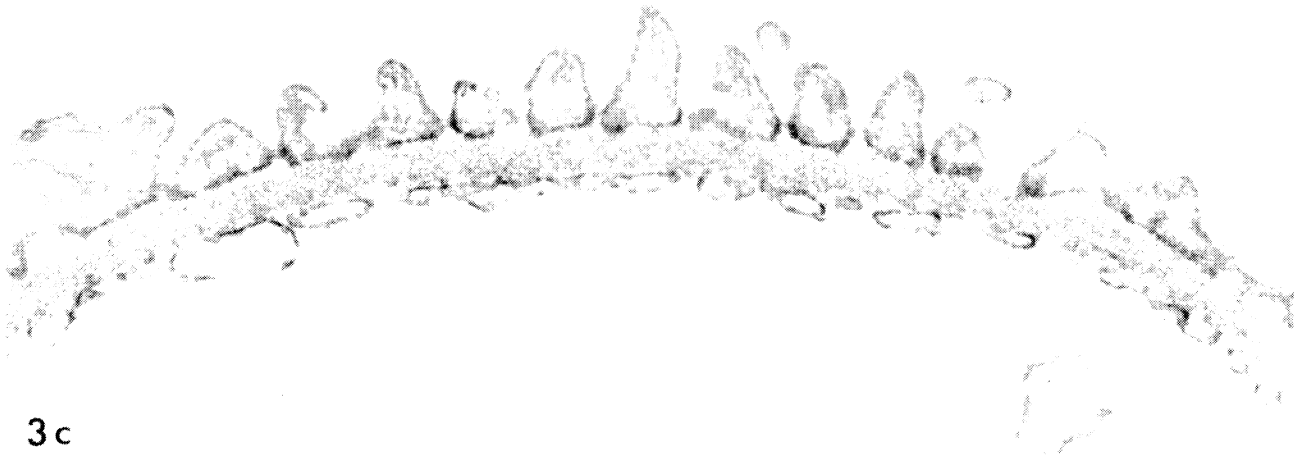


FIG. 3. Binding pattern of monoclonal Ab K12/8 to renal structures. By IIF (*a*) there is a continuous linear staining along basement membranes of glomeruli, tubules, and peritubular capillaries. Minimal mesangial staining could occasionally be observed as well. This pattern is reproduced upon *in vivo* administration of this Ab (*b*); note also intense staining of small artery (*arrow*) and arterole (*arrowhead*). *c*, By immunoelectron microscopy there is a diffuse, light deposition of peroxidase reaction product within the basement membrane. This staining is markedly enhanced (*d*) in areas where the epithelial and endothelial cells have been removed and the Ab had free access to antigenic sites. Compare this pattern with that illustrated in (*e*) which shows a similar area of GBM after incubation with a nonbinding monoclonal Ab of similar class and subclass than K12/8. Figure 3*a* and *b*, ×481; *c*, ×42,000; *d* and *e*, ×28,000.

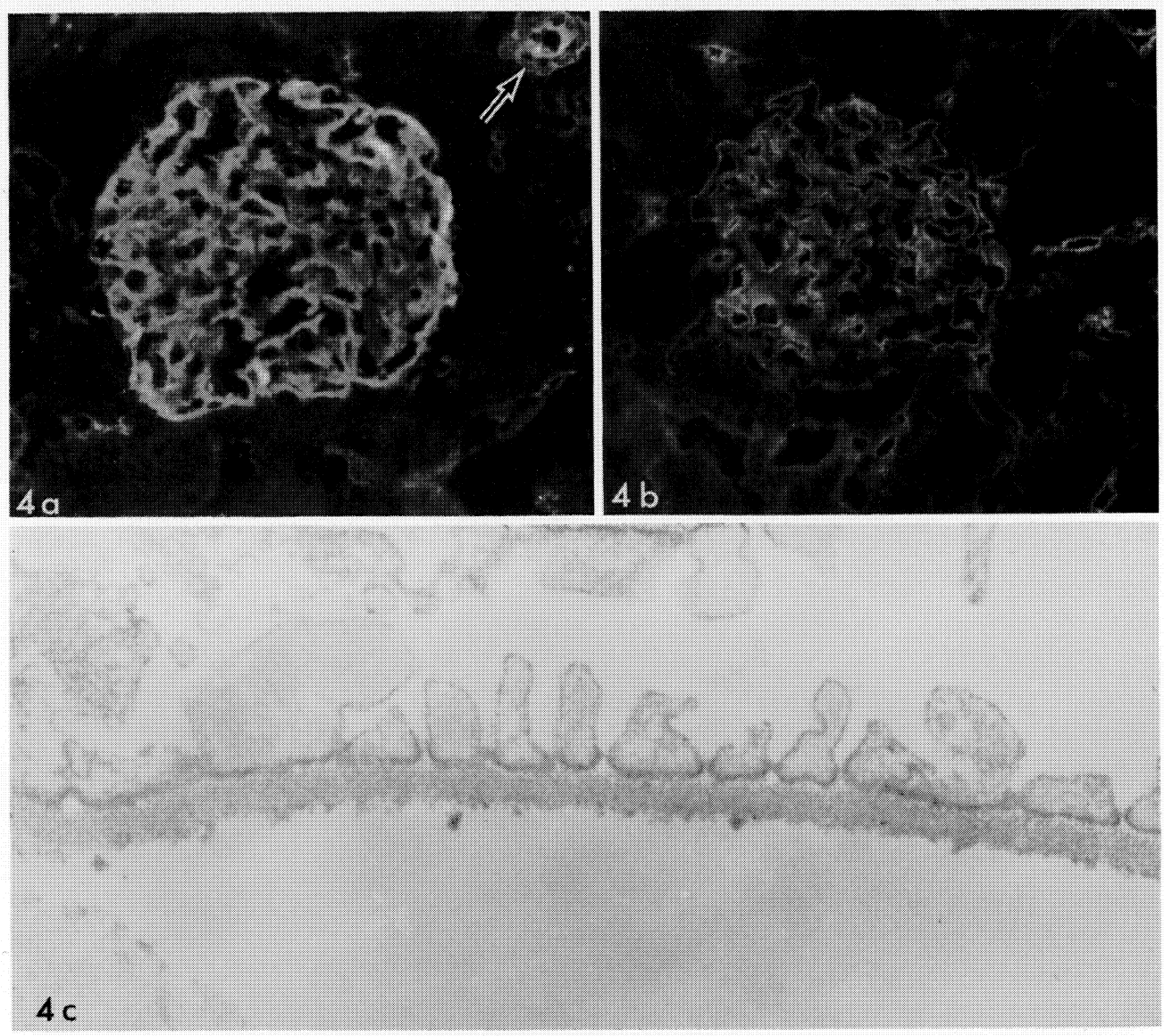


FIG. 4. Binding pattern of monoclonal Ab K12/2 to renal structures. *a*, Ab binds to the glomerular capillary wall, peritubular capillaries, and the basement membranes of vessels (*arrow*) when studied by IIF. *b*, Upon *in vivo* administration of this Ab the glomerular capillary wall and the peritubular capillaries show a linear staining pattern. Tubular basement membrane does not bind this Ab to any significant extent. *c*, Immunoelectron microscopy shows diaminobenzidine reaction product in the GBM. Free epithelial cell membrane is devoid of reaction product. Figure 4*a* and *b*, ×481; *c*, ×42,000.

amples of monoclonal Abs that bind to the basement membranes of glomeruli as well as other epithelial and/or vascular basement membranes of the rat. The patterns of reactivity indicate that these Abs recognize different antigenic determinants. Thus, K12/5 and K12/8 (Fig. 3) bind to GBM, tubular basement membranes, and vascular and epithelial basement membranes in all tissues examined, whereas K12/2 and K17/4 (Fig. 4) are specific for GBM and vascular basement membranes but do not react with the basement membranes of renal tubules or other epithelia (Tables 1 and 2). All four monoclonal Abs show patterns of binding after intravenous administration that are essentially identical with the patterns observed by IIF *in vitro*. However, the pattern obtained after *in vivo* administration of K12/2 or K17/4 results in a linear staining of the GBM that is more delicate than

FIG. 5. Staining patterns of monoclonal Abs reactive with intracytoplasmic components. *a*, K8/8 monoclonal Ab showing an irregular, interrupted linear staining pattern along the glomerular capillary wall. This particular Ab also recognizes an antigenic site in the brush border of the proximal convoluted tubule (*arrows*) and the peritubular capillaries. *b*, Vascular smooth muscle cell staining with monoclonal Ab K17/2. *c*, Smooth muscle cells of the muscularis externa of the small intestine show cytoplasmic staining when reacted with monoclonal Ab K8/8. This binding pattern can easily be distinguished from that of Abs with basement membrane and matrix reactivities like K12/5 as shown in *d*. *3*, Z band staining in striated muscle cells after binding of K9/3 Ab. *f*, K9/7 binding to striated muscle cells. There is irregular, discrete staining of intracytoplasmic elements. *g*, This figure illustrates the binding of K9/3 Ab to the terminal web/brush border of intestinal mucosa cells. Note also the staining of smooth muscle cells within the lamina propria. *h*, Endothelial cells of splenic sinuses of the parafollicular zone showing binding of K17/2 monoclonal Ab. Figure 5*a*, ×332; *b*, ×133; *c*, ×446; *d*, ×664; *e* and *f*, ×484; *g*, ×155; *h*, ×137.

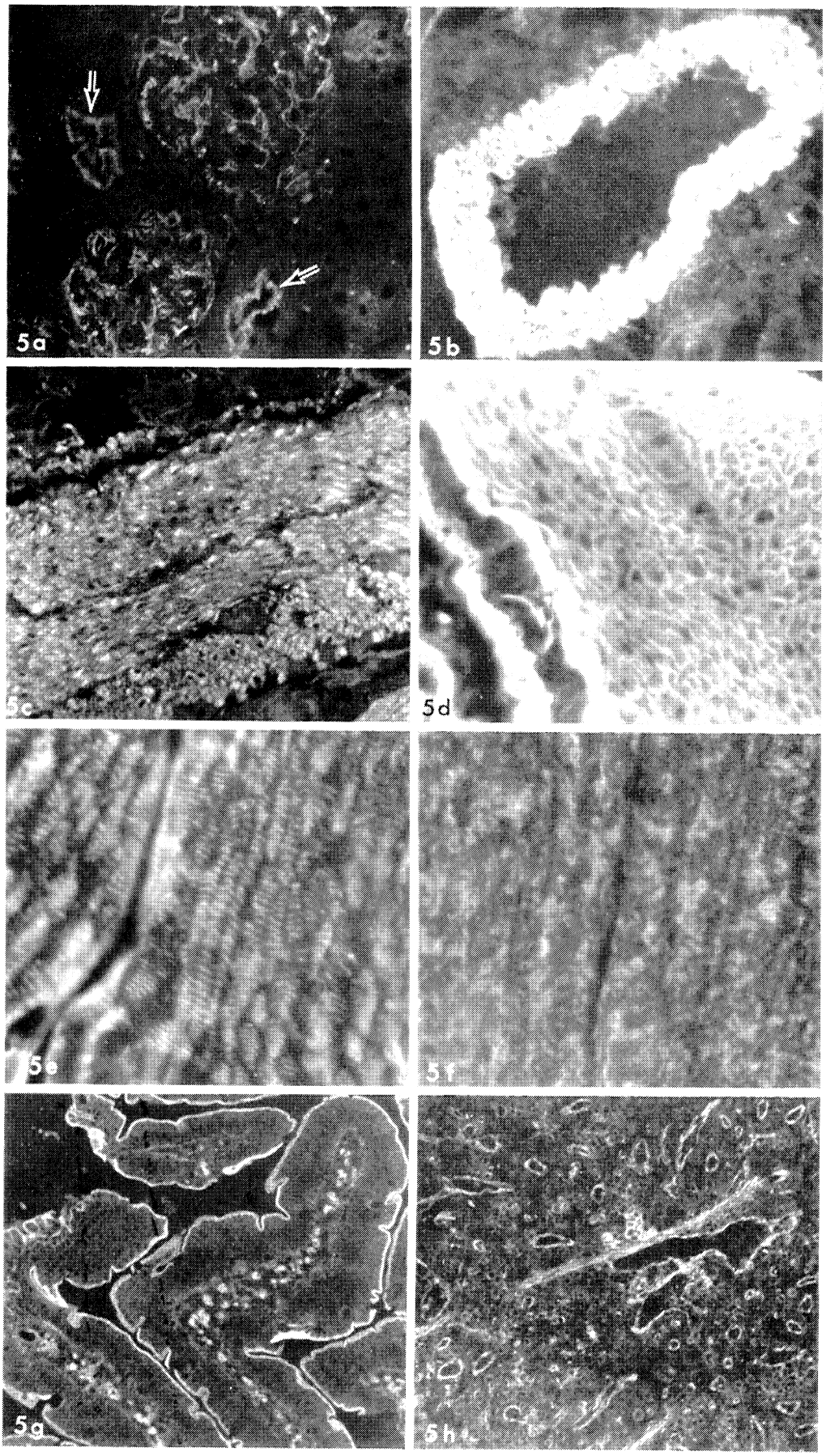


FIG. 5

that observed with IIF (Fig. 4*a* and *b*); this pattern suggests *in vivo* binding to only part of the antigenic sites, most likely the more internally located in the basement membrane and, therefore, more accessible to circulating Abs.

ABS REACTIVE WITH INTRACYTOPLASMIC COMPONENTS

This group of monoclonal Abs (K8/8, K9/3, K9/7, K8/3, K17/2, K17/6, K17/7) reacts with intracellular Ags present in glomerular as well as nonrenal cells; in the glomerulus the binding characteristically results in an interrupted, irregular staining pattern by fluorescence microscopy (Fig. 5*a*). The predominant nonrenal reactivity is with smooth muscle cells of the vascular system (Fig. 5*b*) and the walls of the gastrointestinal and respiratory tract (Fig. 5*c*). Many of them likewise bind to different elements in striated muscle cells (Fig. 5*e* and *f*). The antigenic determinants recognized by some of these Abs are not only found in cells with distinctive contractile functions but are also present in the apical portion of certain epithelial cells, where they appear to be part of either the terminal web or the brush border microvilli (Fig. 5*g* and Tables 1 and 2), and in endothelial cells of the venous sinuses of the spleen (Fig. 5*h*).

DISCUSSION

The ability to prepare monoclonal Abs against a variety of cells and tissues provides a powerful tool for studying the antigenic composition of these tissues and the mechanisms of immunologic injury. In this paper we have described our initial results at producing monoclonal Abs against rat kidneys. Analysis of the binding patterns of different Ags suggests the existence of Ags that cover a spectrum from being highly restricted to widely distributed among tissues of the rat as well as other species.

The Ab with the most restricted specificity we have observed to date is K9/4, which is the only Ab that is specific for an Ag unique to the cell surface of rat glomerular visceral epithelial cells. Recent studies have suggested important physiologic functions for the anionic cell coat of the epithelial cells. These anionic glycoproteins play an important role in the maintenance of the typical morphology of foot processes of podocytes (27) and possibly in cell attachment to the GBM (12) and, hence, in the ultrafiltration process across the glomerular capillary wall (19, 32, 33). An Ab such as K9/4 should be a useful probe for studying the biosynthesis and structure of the epithelial cell coat and may also serve as a tool for the selective isolation and identification of glomerular epithelial cells in culture.

Two other Abs, K6/1 and K6/3, recognize a determinant(s) present on endothelial and epithelial cells as well as in the basement membrane of glomeruli and extrarenal capillaries. This distribution pattern indicates that at least one component of the GBM is also present on the surfaces of different types of glomerular cells, a possibility that has not been experimentally demonstrated previously. Recently, three anionic sialoproteins have been isolated from glomeruli by tryptic digestion; heterologous antisera raised against these proteins react with the surfaces and cytoplasm of epithelial cells and focally with the endothelial cell surface (18). It is clear that the three cell surface-specific Abs that we have produced exhibit staining patterns that are fundamentally different from these heterologous antisera. K6/1 and K6/3 also do not appear to be specific for type V collagen, which is present in endothelial cells and the subendothelial GBM. A protein that is synthesized by both epithelial and endothelial cells and also present in the GBM could be important in maintaining glomerular permeability, and studies using the two monoclonal Abs to investigate this possibility are in progress.

The Abs specific for extracellular basement membranes of vascular and epithelial structures recognize Ags with wide tissue distribution. It is unclear whether these antigenic determinants are present on any of the well-defined components of basement membranes. Types IV and V collagens and laminin have been identified in the basement membranes of glomeruli, tubules, and Bowman's capsule and in the mesangial matrix, and fibronectin is found predominantly in the matrix surrounding mesangial cells (7, 9, 15, 21, 25, 26, 29, 30) and, according to some reports, also in the GBM (6). The apparent absence of binding of the four Abs, K12/2, K12/5, K12/8, and K17/4, to the glomerular mesangium argues against their being specific for type IV or V collagen, laminin, or fibronectin, although this cannot be definitively excluded since the lack of mesangial staining by immunoelectron microscopy could be due to difficulties with penetration of Abs. Furthermore, the *in vivo* binding patterns with the monoclonal Abs are different from the binding of heterologous antisera specific for type IV collagen, laminin, and fibronectin (D. L. Mendrick, H. G. Rennke, R. S. Cotran, T. A. Springer, and A. K. Abbas, unpublished observations). Nevertheless, the specificity of the monoclonal Abs can only be established by isolation and biochemical characterization of the target Ags; these studies are currently in progress.

The binding patterns of the monoclonal Abs with intracytoplasmic reactivity suggest that they recognize ubiquitous determinants present on contractile or cytoskeletal structures. Human glomerular mesangial cells have been shown to possess actomyosin filaments when examined by immunofluorescence microscopy (1, 23, 24); also rat glomerular cells in culture of apparent mesangial origin contract *in vitro* after angiotensin II exposure (2). The striking reactivity of monoclonal Abs directed against contractile structures (*e.g.*, Z bands of skeletal muscle, smooth muscle cells, etc.) with glomerular epithelial cells suggests the possibility of a contractile system in these cells, as well. Such a system could be involved in regulating glomerular hemodynamics and intracapillary hydrostatic pressures, which has been proposed previously on the basis of the anatomical relationship among the podocytes, mesangial cells, and the glomerular capillary wall (3).

Our current efforts are directed at characterizing the Ags recognized by these Abs and at the induction of heterologous disease in the rat with murine monoclonal Abs. The feasibility of such studies is indicated by our

unpublished observations in rats that received serum obtained from mice immunized with rat glomeruli or renal cortex, after an immunization protocol identical with that described earlier for the production of monoclonal Abs. Such rats exhibited proteinuria, glomerular polymorphonuclear influx, and immunoglobulin binding patterns similar to those obtained during the heterologous phase of nephrotoxic serum nephritis using immune rabbit serum (31).

Acknowledgments: We acknowledge the expert technical assistance of Ms. Patricia O'Shea and Ms. Deborah Sandstrom and the skillful secretarial assistance of Ms. Valerie Sherman. Antisera specific for type IV collagen, laminin, and fibronectin were provided by Drs. J. Madri and H. Furthmayer, Yale University School of Medicine.

Date of acceptance: March 22, 1983.

This work was supported by National Institutes of Health Grant 1 RO1 AM27793, Training Grant T32 HL07066-07, and the Milton Fund from Harvard University.

Dr. Rennke is the recipient of a Research Career Development Award from the National Institutes of Health.

Address reprint requests to: Donna L. Mendrick, Ph.D., Pathology Department, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115.

REFERENCES

1. Accinni L, Natali PG, Vassallo L, Hsu KS, De Martino C: Immunoelectron microscopic evidence of contractile proteins in the cellular and acellular components of mouse kidney glomeruli. Cell Tissue Res 162:297, 1975
2. Ausiello DA, Kreisberg JI, Roy C, Karnovsky MJ: Contraction of cultured rat glomerular cells of apparent mesangial origin after stimulation with angiotension II and arginine vasopressin. J Clin Invest 65:754, 1980
3. Barger AC, Herd JA: The renal circulation. N Engl J Med 284:482, 1971
4. Bender BL, Jaffe R, Carlin B, Chung AE: Immunolocalization of entactin, a sulfated basement membrane component, in rodent tissue, and comparison with GP 2 (laminin). Am J Pathol 103:419, 1981
5. Besinger UA, Toyka KV, Anzil AP, Fateh-Moghadam A, Neumeier D, Rauscher R, Heininger K: Myeloma neuropathy: passive transfer from man to mouse. Science 213:1027, 1981
6. Courtoy PJ, Kanwar YS, Hynes RO, Farquhar MG: Fibronectin localization in the rat glomerulus. J Cell Biol 87:691, 1980
7. Daniels JR, Chu GH: Basement membrane collagen of renal glomerulus. J Biol Chem 250:3531, 1975
8. Devulder B, Bardos P, Plouvier B, Martin JC, Muh JP, Tacquet A: Nephrotoxic potency of antisera to three rat glomerular basement membrane glycoproteins. Clin Exp Immunol 32:510, 1978
9. Dixit SN: Isolation and characterization of two α chain size collagenous polypeptide chains C and D from glomerular basement membrane. FEBS Lett 106:379, 1979
10. Graham RC Jr, Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291, 1966
11. Huang F, Kalant N: Isolation and characterization of antigenic components of rat glomerular basement membrane. Can J Biochem 46:1523, 1968
12. Kanwar YS, Farquhar MG: Detachment of endothelium and epithelium from the glomerular basement membrane produced by kidney perfusion with neuraminidase. Lab Invest 42:375, 1980
13. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy (abstr). J Cell Biol 27:137A, 1965
14. Lennon VA, Lambert EH: Myasthenia gravis induced by monoclonal antibodies to acetylcholine receptors. Nature 285:238, 1980
15. Madri JA, Roll FJ, Furthmayr H, Foidart J-M: Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney. J Cell Biol 86:682, 1980
16. Misra RP: The structure of the glomerular basement membrane. Pathobiol Ann 1:323, 1971
17. Nakane PK, Kawaoi A: Peroxidase-labeled antibody: a new method of conjugation. J Histochem Cytochem 22:1084, 1974
18. Nevins TE, Michael AF: Isolation of anionic sialoproteins from the rat glomerulus. Kidney Int 19:553, 1981
19. Olson JL, Rennke HG, Venkatachalam MA: Alterations in the charge and size selectivity barrier of the glomerular filter in aminonucleoside nephrosis in rats. Lab Invest 44:271, 1981
20. Richman DP, Gomez CM, Berman PW, Burres SA, Fitch FW, Arnason BGW: Monoclonal anti-acetylcholine receptor antibodies can cause experimental myasthenia. Nature 286:738, 1980
21. Roll FJ, Madri JA, Albert J, Furthmayr H: Codistribution of collagen types IV and AB_2 in the basement membranes and mesangium of the kidney. J Cell Biol 85:597, 1980
22. Rothbard S, Watson RF: Comparison of reactions of antibodies to rat collagen and to rat kidney in the basement membranes of rat glomeruli. J Exp Med 129:1145, 1969
23. Scheinman JI, Fish AJ, Brown DM, Michael AJ: Human glomerular smooth muscle (mesangial) cells in culture. Lab Invest 34:150, 1976
24. Scheinman JI, Fish AJ, Michael AF: The immunohistopathology of glomerular antigens: the glomerular basement membrane, collagen and actomyosin antigens in normal and diseased kidneys. J Clin Invest 54:1144, 1974
25. Scheinman JI, Foidart J-M, Gehron-Robey P, Fish AJ, Michael AF: The immunohistology of glomerular antigens IV. Laminin, a defined noncollagen basement membrane glycoprotein. Clin Immunol Immunopathol 15:175, 1980
26. Scheinman JI, Foidart J-M, Michael AF: The immunohistology of glomerular antigens. V. The collagenous antigens of the glomerulus. Lab Invest 43:373, 1980
27. Seiler MW, Rennke HG, Venkatachalam MA, Cotran RS: Pathogenesis of polycation-induced alterations ("fusion") of glomerular epithelium. Lab Invest 36:48, 1977
28. Skoza L, Mohos SC: Nephritogenic antigenicity of chromatographic fractions of trypsin and pronase glomerular digests. Proc Soc Exp Biol Med 131:1189, 1969
29. Timpl R, Rohde H, Robey PG, Rennard SI, Roidart J-M, Martin GR: Laminin-A glycoprotein from basement membranes. J Biol Chem 254:9933, 1979
30. Tryggvason K, Kivirikko KI: Heterogeneity of pepsin-solubilized human glomerular basement membrane collagen. Nephron 21:230, 1978
31. Unanue ER, Dixon FJ: Experimental glomerulonephritis. V. Studies on the interaction of nephrotoxic antibodies with tissues of the rat. J Exp Med 121:697, 1965
32. Venkatachalam MA, Cotran RS, Karnovsky MJ: An ultrastructural study of glomerular permeability in aminonucleoside nephrosis using catalase as a tracer protein. J Exp Med 132:1168, 1970
33. Venkatachalam MA, Karnovsky MJ, Cotran RS: Glomerular permeability: ultrastructural studies in experimental nephrosis using horseradish peroxidase as a tracer. J Exp Med 130:381, 1969
34. Yaar M, Foidart JM, Brown KS, Rennard SI, Martin GR, Liotta L: The Goodpasture-like syndrome in mice induced by intravenous injections of anti-type IV collagen and anti-laminin antibody. Am J Pathol 107:79, 1982