

A Nephritogenic Rat Monoclonal Antibody to Mouse Aminopeptidase A. Induction of Massive Albuminuria after a Single Intravenous Injection

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Summary

Antibodies directed against antigens present on renal epithelial cells can cause membranous glomerulonephritis in experimental animals, which closely resembles the human form of this disease. However, most antibodies produced so far fail to cause the persistent and severe proteinuria that is seen in humans. In our search for new antibodies of this kind, we have now produced a monoclonal antibody (mAb) against mouse aminopeptidase A, a hydrolase that is present in the mouse kidney. The mAb (ASD-4) was prepared by fusion of mouse myeloma cells with splenocytes of Lou rats immunized with brush border (BB) membranes from mouse kidneys. ASD-4 is of the IgG1 subclass and reacts with a 140-kD protein as demonstrated by immunoprecipitation on radiolabeled BB membranes. In indirect immunofluorescence and immunoelectronmicroscopy of normal mouse kidneys, ASD-4 was diffusely present on the BB of the S1 and S2 segments of the proximal tubules, and on the cell membranes of the glomerular visceral epithelia. It also bound to cell membranes of nonglomerular endothelia, smooth muscle cells of arteries, and juxtaglomerular cells. After injection of ASD-4 into normal mice, an immediate homogeneous binding to the capillary wall was seen that gradually changed into a fine granular pattern after 1 d. This glomerular binding was followed by binding to the BB and basolateral membranes of the convoluted proximal tubules. Immediately after injection of ASD-4, a dose-dependent albuminuria occurred that lasted for at least 16 d. ASD-4 is thus a new rat mAb against a well-defined renal epithelial antigen that causes not only membranous glomerulonephritis after a single injection in the mouse, but also severe albuminuria.

One of the mechanisms that can result in immune complex formation in the glomerular capillary wall and eventually can lead to functional and morphologic glomerular damage is the interaction of antibodies with intrinsic antigens on the glomerular visceral epithelial or endothelial cells (1-3). The most extensively studied example of such a mechanism is the Heymann nephritis in the rat, an experimental model of membranous glomerulonephritis, in which the glomerular damage and ensuing proteinuria is initiated by the binding of antibodies to a structural protein with a molecular mass of 330 kD located at discrete sites on the cell membranes of the glomerular visceral epithelial cells (4-6). These findings from the Heymann nephritis in the rat have stimulated many investigators to characterize other cell membrane-bound antigens that might play a role in the formation of immune

complexes in the glomerulus and the induction of glomerular lesions and proteinuria.

The hydrolase dipeptidyl peptidase IV (DPP IV)¹ is one of these antigens that has been reported to be involved in the induction of an immune complex glomerulonephritis (7-13). Injection of monoclonal or polyclonal antisera against DPP IV into rats or mice results in an immediate, transient glomerular binding, but induces only a mild proteinuria of short duration (7, 9, 11). An autologous phase with the formation of small subepithelial immune deposits develops after injection of polyclonal, but not of monoclonal anti-DPP IV antibodies. However, in this phase there is no enhanced glomerular permeability (11). In recent years, several other cell

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¹ Abbreviations used in this paper: APA, aminopeptidase A; APN, aminopeptidase N; BB, brush border; DPP IV, dipeptidyl peptidase IV; GBM, glomerular basement membrane; IEM, immuno-electron microscopy; IF, immunofluorescence; IP, immunoprecipitation; LM, light microscopy; S4B, Sepharose 4B.

membrane-bound proteins have been isolated from glomeruli of rats and rabbits (14–23), but mAbs against only two of these can cause a glomerulonephritis accompanied by some degree of proteinuria in rats (17, 21).

Until now, there are no data on well-characterized glomerular cell membrane-bound proteins that might be involved in an immune complex glomerulonephritis with massive albuminuria in mice. In this study, we described a rat mAb against mouse aminopeptidase A (APA), a hydrolase that, like DPP IV, is prominently present on the cell membranes of glomerular visceral epithelial cells and brush border (BB) of proximal tubules. It is the first mAb that can induce a severe albuminuria in mice after a single intravenous injection.

Materials and Methods

Materials. Aquamount was purchased from BDH Chemicals Ltd., Poole, UK; Sephadex G-25, Sepharose 4B-coupled protein A, Sepharose 4B, and DEAE-Sephacel from Pharmacia, Uppsala, Sweden; paraplast from Amstelstad, Amsterdam, The Netherlands; 96-well tissue culture clusters from Costar, Cambridge, MA; Hepes-buffered RPMI 1640 from M. A. Bioproducts, Bethesda, MA; FCS from Gibco Laboratories, Grand Island, NY; AP-15 filter from Millipore Continental Water Systems, Bedford, MA; isotyping kit for rat mAbs from Serotec, Oxford, UK; aprotinin (Trasylol) from Bayer, Leverkusen, Germany; L-glutamic acid- α -7-amido-4-methylcoumarin (Glu.AMC) and glycyl-L-proline-7-amino-4-methylcoumarin-HBr (Gly.Prol.AMC) from Bachem, Bubendorf, Switzerland; L-alanine-7-amido-4-methylcoumarin (Ala.AMC) from Serva GMBH and Co., Heidelberg, Germany; protein A-agarose from Sigma Chemical Co., St. Louis, MO; FITC-labeled rabbit anti-rat IgG from Cappel-Organon Technika NV, Turnhout, Belgium; FITC-labeled goat anti-mouse C3 from Nordic, Tilburg, The Netherlands; FITC-labeled sheep anti-mouse IgG (heavy and light chains), F(ab')₂ fragment from Sigma Chemical Co.; peroxidase-labeled sheep anti-rat IgG, Fab fragment, from Sanbio, Uden, The Netherlands; Iodobeads from Pierce Chemical Co., Rockford, IL; sodium-iodide-125 (¹²⁵I) from Amersham, Buckinghamshire, UK; high molecular weight standards, and coomassie brilliant blue R-250 from Bio-Rad Laboratories, Richmond, CA.

Animals. Male Lou rats used for the production of mAb were obtained from the Harlan Olac Ltd., Blackthorn, Bicester, UK. Female and male BALB/c mice weighing 20–25 g and aged 3–6 mo, were originally obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/c, *nu/nu* mice were bred in the breeding facility of the Central Laboratory of Animals of our University. All mice were kept in our animal laboratory by continuous brother-sister matings.

Preparation of BB Suspensions from Mouse Kidneys. An enriched suspension of mouse renal BB membrane vesicles from proximal tubular epithelial cells was prepared from BALB/c kidneys according to the method of Malathi et al. (24), using a 2-mM Tris-HCl buffer, pH 7.2, containing 50 mM mannitol, the protease inhibitors EDTA (20 mM), PMSF (1 mM), benzamidine (1 mM), Trasylol (10 U/ml), and 0.02% NaN₃. To obtain a BB preparation enriched in integral membrane proteins that served as immunogen for the production of mAb, the crude BB fraction was solubilized with the non-ionic detergent Triton-X-114 (2%) in 50 mM Tris-HCl at 4°C for 30 min and subjected to Triton X-114 phase separation at 37°C for 20 min followed by centrifugation at 300 g for 5 min (25). In the immunoprecipitation procedure a BB fraction solubilized

with 2% Triton X-100 in 50 mM Tris-HCl at pH 7.2, without the protease inhibitors, was used. For the fluorimetric enzyme assay BB membrane vesicles were solubilized with 1% Doc in 50 mM Tris-HCl at pH 8.5, also without protease inhibitors for 30 min at 4°C, and subsequently centrifuged at 100,000 g for 1 h at 4°C. The supernatant was then dialyzed overnight at 4°C against 0.1% Doc. Protein concentrations were determined by the method of Lowry et al. (26).

Preparation of mAb to APA. A male Lou rat was immunized intraperitoneally with 1 ml of the detergent phase of the Triton X-114 extract of mouse BB and boosted by three intraperitoneal injections. 3 d after the last booster injection, spleen cells from the Lou rat were fused with SP 2/0 mouse myeloma cells by the procedure of Köhler and Milstein (27), plated in 96-well tissue culture plates with Hepes-buffered RPMI 1640, containing 10% decomplexed FCS, gentamycin (40 µg/ml), 1 mM L-glutamine, 1 mM sodium pyruvate, 10% supernatant of a human umbilical vein cell culture, supplemented with HAT. Supernatants of wells containing growing hybridomas were tested for antibodies to antigens, present on both BB and glomerular epithelial cells, by indirect immunofluorescence (IF) on acetone-fixed cryostat sections from normal BALB/c kidneys. Selected hybridomas producing antibodies of the desired specificity were expanded after HAT selection in conventional culture medium, and cloned several times by limiting dilution until all subclones showed the same reactivity pattern in the indirect IF screening procedure. 10 mAbs (listed in Table 1) were subsequently produced in larger amounts by intraperitoneal inoculation of the cloned hybridomas in BALB/c, *nu/nu* mice pretreated with pristane. Ascites samples were purified by ammonium sulfate precipitation and tested for their nephritogenicity and their effect on the APA activity of mouse BB vesicles. The mAb coded as ASD-4 was selected for more extensive studies after further purification by chromatography on DEAE-sephacel.

The IgG content of the antibody preparations was measured by radial immunodiffusion (28). IgG subclasses were determined with an isotyping kit for rat mAb according to the instructions of the manufacturer (Serotec). The test system is based on rat cell agglutination using highly specific antibodies directed to isotypes of rat Igs attached to sheep red cells (29). The purified ASD-4 was subjected to agarose isoelectric focusing to determine its isoelectric spectrotyping.

Immunoprecipitation. Mouse renal BB membrane vesicles (23 mg/ml) solubilized by Triton X-100 were radiolabeled with ¹²⁵I using Iodobeads as a coupling reagent (30). Free ¹²⁵I was removed by Sephadex G-25 chromatography. 100 µl of protein A-agarose beads were incubated with 50 µl of an ammonium sulfate precipitation-purified rabbit anti-rat Ig for 4 h at room temperature. Supernatants of three growing hybridomas selected by indirect IF and normal rat serum as a control were added to these beads and incubated overnight at 4°C under constant agitation. As additional controls a goat anti-rat gp90 antiserum (kindly provided by Dr. E. de Heer, Department of Pathology, University Hospital Leiden, Leiden, The Netherlands) and normal goat serum were added to protein A-agarose beads loaded with rabbit anti-goat Ig. After several washes with immunoprecipitation (IP) buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.1% [vol/vol] Triton X-100, 0.02% [wt/vol] SDS, and 10 U/ml Trasylol), pH 7.4, 50 µl of the radiolabeled mouse BB suspension was added to these beads and incubated for 2 h at room temperature. The beads were then washed over an AP-15 filter with the IP buffer several times. The beads were gently removed from the filter and heated in a SDS sample buffer (2.3% SDS, 200 mM dithiothreitol, 10% glycerol, 60 mM Tris-HCl buffer, pH 6.8) for 5 min and spun down for 5 min in

a microfuge. The immunoprecipitated proteins were analyzed on SDS-PAGE in 5–18% acrylamide gradients according to Laemmli (31). For autoradiography, the gels were dried and exposed at -70°C with preflashed x-ray film (Kodak XAR-5).

Fluorimetric Enzyme Assay. The specificity of ASD-4 for the hydrolase APA was determined by a solid phase immunodepletion procedure (16). The purified mAb was first coupled to cyanogen bromide-activated Sepharose 4B (S4B) according to the instructions of the manufacturer. Briefly, 5 mg of the selected mAb in 1 ml of 0.1 M carbonate buffer, pH 8.4, containing 0.5 M NaCl was added per ml of S4B. Residual binding sites were saturated with 1 M ethanolamine. Then, 4 mg of a BB suspension solubilized by Doc and dissolved in 5 ml buffer was incubated for 18 h at 4°C under constant stirring with 1 ml of S4B coupled to the mAb. A control BB suspension was prepared by performing a similar incubation of S4B beads to which no mAb had been coupled. The supernatant was removed and the beads were washed several times with buffer. Elution of the antigen bound to 1 ml of beads was performed with 5 ml of 50 mM diethylamine, suspended in 0.1% Doc, at pH 12 for 5 min. The eluted proteins were neutralized with 2 M Tris, pH 7.4. The enzymatic activities for APA, aminopeptidase N (APN), and DPP IV were measured fluorimetrically in the control BB suspension, the immunodepleted suspension, and the eluate using Glu.AMC, Ala.AMC, and Gly.Pro.AMC as specific and sensitive substrates for APA, APN, and DPP IV, respectively (32). For the determination of APA activity, the three fractions and the substrate were solubilized in 0.1 M Tris-HCl buffer, containing 1.25 mM CaCl_2 , pH 7.0. For APN activity, 0.1 M Tris-HCl buffer, pH 7.0, and for DPP IV activity 0.1 M phosphate buffer, pH 8.0, were used as solutions. 10 μl of one of the three fractions was incubated with 100 μl of one of the three substrates (2×10^{-4} M) for 20 min at 37°C . The reaction was stopped by the addition of 890 μl of 0.2 M Tris-NaOH, pH 11. The enzyme activities were determined by the fluorescence of 7-amino-4-methylcoumarin cleaved from the substrates (32). The fluorescence was measured at 370 nm (excitation) and 460 nm (emission) using a luminescence spectrometer (LS5; Perkin-Elmer, Norwalk, CT). The enzyme activities of the various fractions are expressed either as nanokatal/ml (1 nanokatal being the amount of enzyme that converts 1 nmol of substrate per second under the given assay conditions) or as percentages of the activities present in the control BB suspension.

Effect of Various mAbs on APA Activity of BB Vesicles. The ability of the antibodies to block the APA activity on BB vesicles was tested in an inhibition enzyme assay. 20 μl of a BB preparation, containing 1 μg of protein, was incubated with 20 μl of each of the mAbs, containing 5–10 μg IgG, for 10 min at room temperature. Then, 40 μl of the substrate Glu.AMC (5×10^{-4} M) was added to the suspension, which was incubated for another 30 min at 37°C . The whole preparation was then dissolved in 0.1 M Tris-HCl, pH 7.0, with 1.25 mM CaCl_2 . Hydrolysis of the substrate was stopped by adding 920 μl of 0.2 M Tris. NaOH, pH 11, and the enzyme activity was measured as described before. The enzyme activity of the starting BB suspension was expressed in nkatal/ml and the inhibition of the APA activity of the BB vesicles by the mAb is given as a percentage of residual activity.

In Vivo Binding of the mAb. Groups of five female BALB/c mice were injected intravenously via the tail vein with 0.5, 1, 2, 4, and 8 mg of mAb ASD-4, respectively. Albuminuria, as a sign of glomerular protein leakage, was measured in 18-h urine samples collected 1 d before, and at days 1, 4, 8, and 16 after injection of the mAb. During their confinement in individual metabolic cages, the mice received only tap water ad libitum (33). Pathological al-

buminuria was defined as a value greater than the normal mean plus 2 SD. Urinary albumin-concentrations were measured by radial immunodiffusion in 18-h urine samples, using a goat antiserum against mouse albumin (28, 33). The upper limit of the physiologic albuminuria for females of this mouse strain is 86 μg of albumin excreted during an 18-h period. To determine the immunohistological lesions, female BALB/c mice were injected intravenously with 4 mg ASD-4. Groups of three mice were killed at days 1, 4, 8, and 14, and their kidneys were removed and processed for light microscopy (LM), IF, EM, and immuno-electron microscopy (IEM).

The in vivo binding and the nephritogenic capacity of nine other mAbs, listed in Table 1, were studied by injecting 10 mg of mAb, purified by ammonium sulfate precipitation and dissolved in 0.7 ml of PBS, intravenously into groups of three female BALB/c mice. Albuminuria was measured at day 1, after which the kidneys were removed and processed for IF.

Light Microscopy, Immunofluorescence, and Electron Microscopy. Kidney fragments were fixed in Bouin's solution, dehydrated, and embedded in paraplast, and 2- μm sections were stained with hematoxylin and eosin, Periodic Acid Schiff, and Silver methenamine, as described earlier (11). For IF, kidney fragments were snap frozen in liquid nitrogen, and 2- μm acetone-fixed cryostat sections were stained with FITC-labeled rabbit anti-rat IgG, absorbed with 40 $\mu\text{l}/\text{ml}$ normal mouse serum, FITC-labeled sheep anti-mouse IgG, absorbed with 40 $\mu\text{l}/\text{ml}$ normal rat serum, and FITC-labeled goat anti-mouse C_3 for 1 h. After rinsing in PBS, all sections were embedded in Aquamount. Binding of the mAb ASD-4 to normal mouse kidney was visualized with FITC-labeled rabbit anti-rat IgG in an indirect IF procedure. The sections were examined in a fluorescence microscope equipped with a Ploemopak epiillumination (Leitz Letzlar GmbH, Wetzlar, Germany), and the staining intensity was recorded semiquantitatively on a scale from 0 to 4+ as described before (11). For EM, small pieces of cortex were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer, pH 7.4, for 4 h at 4°C , and washed in the same buffer. The tissue fragments were both fixed in phosphate-buffered 2% OsO_4 for 2 h, dehydrated, and embedded in Epon 812. Ultrathin sections were cut in an ultratome (LKB Instruments, Bromma, Sweden) and stained with 4% uranyl acetate for 45 min and with lead citrate for 2 min at room temperature. The sections were examined in an electron microscope (300; Philips, Eindhoven, The Netherlands).

Immuno-electron Microscopy. The localization of the antigen in a normal BALB/c kidney was examined by indirect IEM and the binding of the mAb to renal structures after intravenous injection into normal BALB/c mice by direct IEM using immunoperoxidase labeling on 20- μm frozen sections. The left kidney of a BALB/c mouse was first perfused with PBS, pH 7.4, for 5 min and subsequently with a mixture of periodate, lysine, and 2% paraformaldehyde (PLP) for 10 min (12). The kidney was removed and small cortical pieces were immersed for an additional 3 h. After rinsing several times in PBS, the fragments were cryoprotected by immersion in 2.3 M sucrose, pH 7.2, for 1 h, and then frozen in liquid nitrogen. The mAbs and all labeled antisera were diluted in PBS containing 1% BSA. 20- μm thick sections were rinsed in PBS for 1 h, then incubated with the mAb for 18 h at 4°C followed after several washes with PBS by incubation with a peroxidase-labeled rabbit anti-rat IgG, diluted 1:20, for 1.5 h. For the presence of mAb bound to the kidney after intravenous injection, only the latter incubation step with the peroxidase-labeled rabbit anti-rat IgG was performed. After three washes in PBS, the sections were incubated in diaminobenzidine (DAB) medium containing 0.6% Tris for 10 min, followed by DAB with addition of 0.003% H_2O_2 for 7 min.

The sections were washed in distilled water, postfixed in 0.1 M phosphate-buffered 2% OsO₄ for 30 min at room temperature, dehydrated, and embedded in Epon 812. Thin sections were prepared on an ultratome (LKB Instruments) and examined unstained in the electron microscope.

Statistical Analysis. For statistical analysis, Wilcoxon's rank sum test was used. *p* values <0.05 were regarded as significant. All values are expressed as means ± SD.

Results

Characterization of mAbs. Eight antibodies that showed an identical localization in the kidney by indirect IF, were directed against APA (Table 1). One mAb (ASD-17) was directed to a yet unidentified component of the glomerular and tubular basement membranes (GBM; TBM), while another mAb (ASD-43) was specific for DPP IV (data not shown). The more extensively studied ASD-4 was of the IgG1 subclass and showed three distinct bands of isotypes with *p*I of 6.4, 6.7, and 6.9 by IEF (data not shown). The molecular mass of the antigen recognized by ASD-4 was determined by immunoprecipitation on radiolabeled mouse BB. As shown in Fig. 1, ASD-4 (lane 6) immunoprecipitated one band with an apparent molecular mass of 140 kD. Two other mAbs, coded ASD-2 and ASD-3, with an identical renal localization in IF as ASD-4, also bound to a 140-kD protein

Table 1. Characteristics of Eight mAbs against Mouse Aminopeptidase A

Code mAb*	Subclass	Albuminuria at day 1 [†]	FEA [‡]
			%
ASD-2	IgG1	18,079 ± 7,194 (3)	8
ASD-3	IgG1	25,011 ± 11,249 (3)	8
ASD-4	IgG1	18,033 ± 5,564 (3)	10
ASD-37	IgG2a	96 ± 10 (3)	18
ASD-38	IgG2b	93 ± 31 (3)	116
ASD-39	IgG2a	160 ± 121 (3)	22
ASD-41	IgG2a	78 ± 35 (3)	93
ASD-44	IgG2a	140 ± 144 (3)	105
ASD-17	IgG1	74 ± 9 (3)	105
ASD-43	IgG2b	107 ± 20 (3)	95
PBS	-	104 ± 64 (5)	-

* All mAbs were purified by ammonium sulfate precipitation. ASD-17 is directed to an as yet unidentified component of GBM/TBM, and ASD-43 is directed to DPP IV.

[†] 10 mg of mAb dissolved in 0.7 ml of PBS or 0.7 ml PBS alone was injected intravenously into BALB/c mice. Numbers of mice appear between parentheses. Data are micrograms of albumin per 18 h.

[‡] Residual APA activity of BB vesicles after preincubation with the mAb in the fluorimetric enzyme assay (FEA). Original activity in the preparation was 9.4 nkatal/ml.

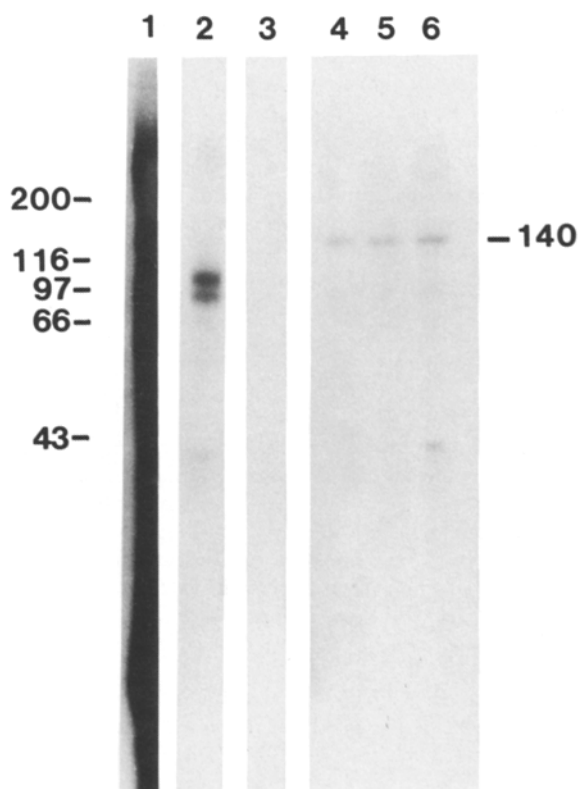


Figure 1. Autoradiogram of the immunoprecipitation analysis of radiolabeled mouse renal BB after SDS-PAGE (5-18%). Lane 1, whole BB preparation; lane 2, polyclonal anti-mouse DPP IV; lane 3, normal goat serum; lanes 4 (ASD-2) and 5 (ASD-3), two different rat mAb directed to APA; lane 6, ASD-4, a rat mAb against APA described in the study. The numbers in the figure indicate the molecular mass of the markers.

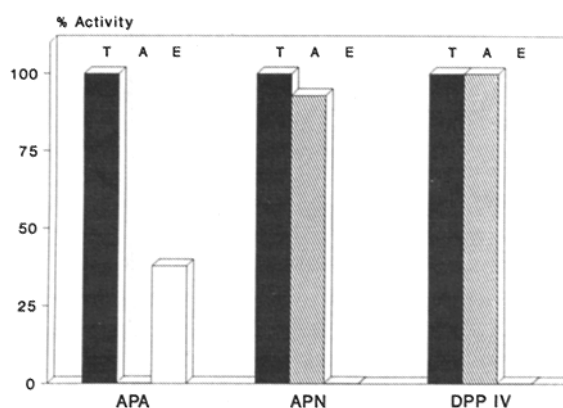


Figure 2. Enzymatic activities of APA, APN, and DPP IV measured in three fractions using a fluorimetric enzyme assay. A Doc-solubilized BB fraction (T; filled bars), a BB fraction immunodepleted with ASD-4 coupled to Sepharose-4B beads (A; hatched bars), and a fraction eluted from the beads (E; open bar). Enzymatic activities are given as percentages of the activities in the starting material (100%). The original activities were 5.6, 34.7, and 12.3 nkatal/ml for APA, APN, and DPP IV, respectively.

Table 2. The Presence of Aminopeptidase A in the Kidney of a Normal BALB/c Mouse

Renal structure	Staining intensity
Glomerulus	
Visceral epithelium	+++
Endothelium	-
Juxtaglomerular cells	+
Proximal tubule	
Brush border	S1 S2 S3
S1	+++
S2	+++
S3	±
Peritubular capillaries	
Cortex	+
Medulla	++
Arteries	
Muscle cell membranes	+

Staining intensity was detected with ASD-4 using the indirect immunofluorescence technique.

(lanes 4 and 5). A low molecular mass band is also seen in lane 6, probably as a degradation product. A polyclonal antiserum against mouse DPP IV precipitated two bands, one with a molecular mass of 108 kD, and the other with a molecular mass of 80 kD (lane 2). Normal rat or goat serum (lane 3) did not precipitate any protein from the BB suspension.

Specificity of the mAb The specificity of ASD-4 was determined by an immunoadsorption procedure using Sepharose-4B beads coated with ASD-4 to deplete the specific antigen from the BB suspension. As shown in Fig. 2, enzymatic activities could be detected in the Doc-solubilized BB preparation for APA, APN, and DPP IV using specific substrates in the fluorimetric enzyme assay. APA activity was selectively depleted from the starting material by the immunoadsorbent, which did not influence the enzymatic activities for APN and DPP IV. A basic elution step could recover ~38% of the enzymatic activity for APA, but not for DPP IV and APN. The incomplete recovery of the enzymatic activity for APA by the elution is most likely caused by the partial inactivation of APA due to the high pH used for the elution procedure. These results, which were confirmed in two additional experiments, strongly suggest that the antigen to which ASD-4 bound has an enzymatic activity of APA.

Localization of APA in Normal Mouse Kidneys. By indirect IF, APA could be detected in several defined areas of the mouse kidney (Table 2). ASD-4 strongly stained the visceral epithelium of the glomerulus and the BB of the convoluted proximal tubules (Fig. 3 A). In addition, it stained to a lesser extent the juxtaglomerular cells (Fig. 3 A), the BB of the straight parts of the convoluted proximal tubules, the endothelial cells of the peritubular capillaries, especially those located in the medulla (Fig. 3 B), and the cell membranes of the smooth muscle cells of arteries. Minimal staining could also be seen in the basal parts of some cells of the convoluted proximal tubules. The endothelial cells of arteries and veins were negative.

By indirect IEM, the antigen was homogeneously present on the cell membranes of the glomerular visceral epithelia.

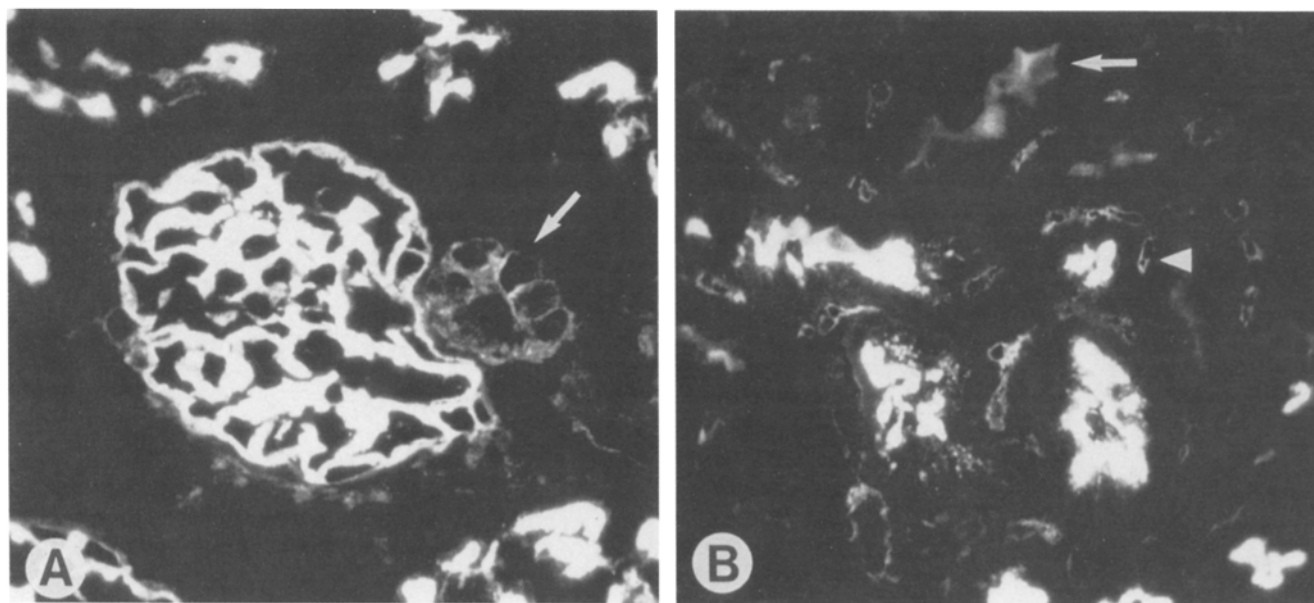


Figure 3. Indirect immunofluorescence of a normal BALB/c kidney incubated with ASD-4. (A) Strong homogeneous fluorescence along the capillary wall of the glomerulus and the BB of the proximal tubules is present. Fine granular staining of the juxtaglomerular cells can also be seen (arrow) ($\times 800$). (B) Strong binding to the BB of the convoluted part and faint binding to the straight part (arrow) of the proximal tubules can be observed. Endothelia of the peritubular capillaries are also positive (arrowhead) ($\times 530$).

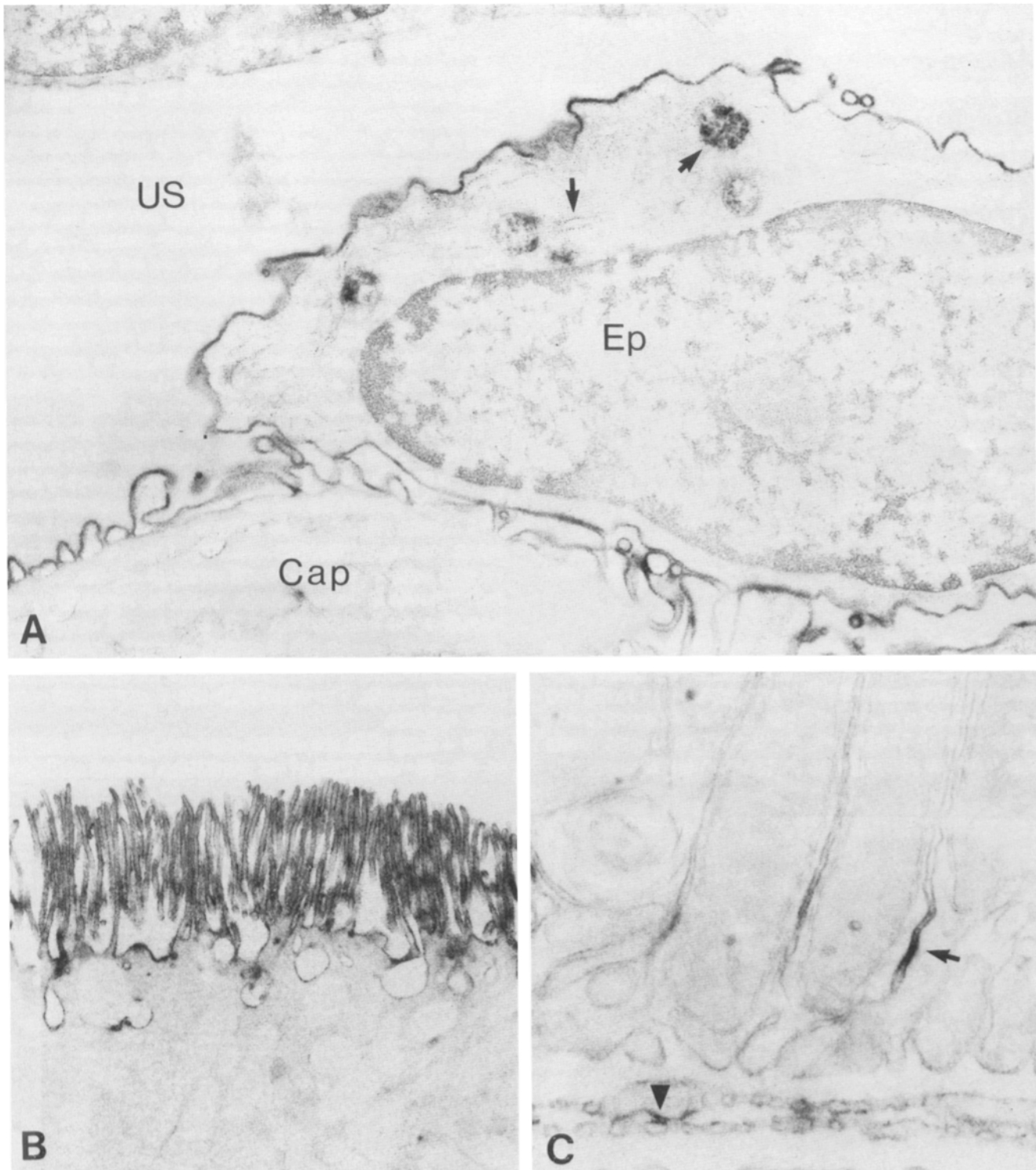


Figure 4. Indirect immuno-electron microscopy of a normal BALB/c kidney incubated with ASD-4. A-C Immunoperoxidase labeling. (A) A higher magnification of a capillary loop with the homogeneous staining of the cell membranes of the visceral epithelial cell and the staining of some multivesicular bodies and the endoplasmic reticulum (*arrows*) ($\times 20,600$). Cap, capillary lumen; Ep, epithelial cell; US, urinary space. (B) Homogeneous staining of the BB of convoluted proximal tubules and some vesicles. ($\times 10,175$). (C) Discrete foci of staining of the basolateral membranes of the convoluted proximal tubule (*arrow*), and some staining of the endothelium of a peritubular capillary (*arrowhead*) ($\times 44,000$).

Table 3. Albuminuria in BALB/c Mice at Day 1 after Intravenous Injection of ASD-4

Dose	Albuminuria*	
mg		
-	34 ± 23	(30) [‡]
0.5	37 ± 20	(5)
1	66 ± 42	(5)
2	1,133 ± 502 [§]	(5)
4	4,555 ± 1,920 [§]	(5)
8	12,035 ± 7,543 [§]	(5)

* Data show micrograms albumin per 18 h.

‡ Numbers of mice are in parentheses.

§ Significant different from untreated mice, ($p < 0.02$).

but not on endothelial or mesangial cells (Fig. 4 A). In addition, it was strongly and diffusely present on the BB (Fig. 4 B) and focally on the basal lateral cell membranes of the convoluted proximal tubules (Fig. 4 C). The presence of the antigen on the endothelial cells of the peritubular capillaries (Fig. 4 C), muscle cells of arteries, and to a lesser extent on the BB of the straight parts of the proximal tubules, was also confirmed by IEM. Intracellularly, the antigen could be demonstrated in small or multivesicular bodies, in the endoplasmic reticulum of the glomerular epithelial cells (Fig. 4 A), and on the cell membranes of small vesicles just below or pinching off the base of BB of the convoluted proximal tubules (Fig. 4 B). The distal tubules, collecting ducts, loops of Henle, and all basement membranes were negative.

Induction of Albuminuria in Normal BALB/c Mice. When BALB/c mice were injected intravenously with increasing (0.5–8 mg) amounts of ASD-4, an albuminuria could be induced at day 1 with doses >1 mg of mAb, which was massive with a dose of 8 mg (Table 3). The albuminuria remained

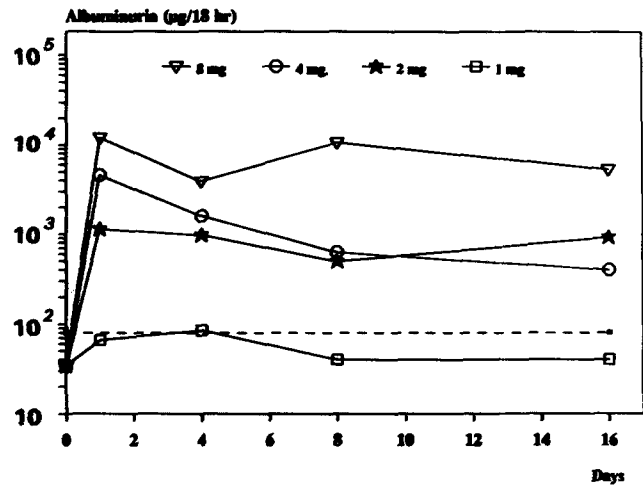


Figure 5. Albuminuria in BALB/c mice after injection of 1, 2, 4, and 8 mg of ASD-4. Albuminuria is given as total amount of albumin in 18-h urine samples (mean). The dashed line represents the upper limit of the physiologic albuminuria.

high and had not returned to normal values 16 d after the injection of the mAb (Fig. 5). Mice injected with doses >8 mg became severely ill at the end of their confinement in the metabolic cages at day 1 and most did not survive. Injection of 8 mg of a rat mAb with the same isotype as ASD-4 and directed to an as yet unidentified antigen present on glomerular epithelia and endothelia resulted in strong glomerular binding but not in a pathologic albuminuria (data not shown).

In Vivo Binding of ASD-4 to Renal Structures. Injection of 4 mg of ASD-4 into normal mice led to an immediate binding to the glomerular capillary wall in a pattern that was homogeneous as seen by direct IF at 1 h (Table 4, Fig. 6 A). This prominent staining pattern changed gradually into a more granular, membranous pattern after 1 d. At day 14, the rat mAb was present in fine granules along the capillary wall

Table 4. Immunofluorescence Findings in BALB/c Mice after Injection of 4 mg ASD-4

Time	Glomerulus			Proximal tubule	
	Ra.IgG	M.IgG	M.C3	BB (Ra.IgG)	BL (Ra.IgG)
1 h	++h	-	-	+	-
4 h	+++h	-	-	++	-
1 h	+++g	-	-	++	+
3 d	++g	-	-	-	+++
8 d	+++g	±	-	-	+++
14 d	+++g	++	±	-	-

BB, brush border; BL, basolateral membranes; Ra, rat; M, mouse; h; homogeneous staining pattern, g; granular staining pattern.

* Only a few granules in the capillary wall of some glomeruli.

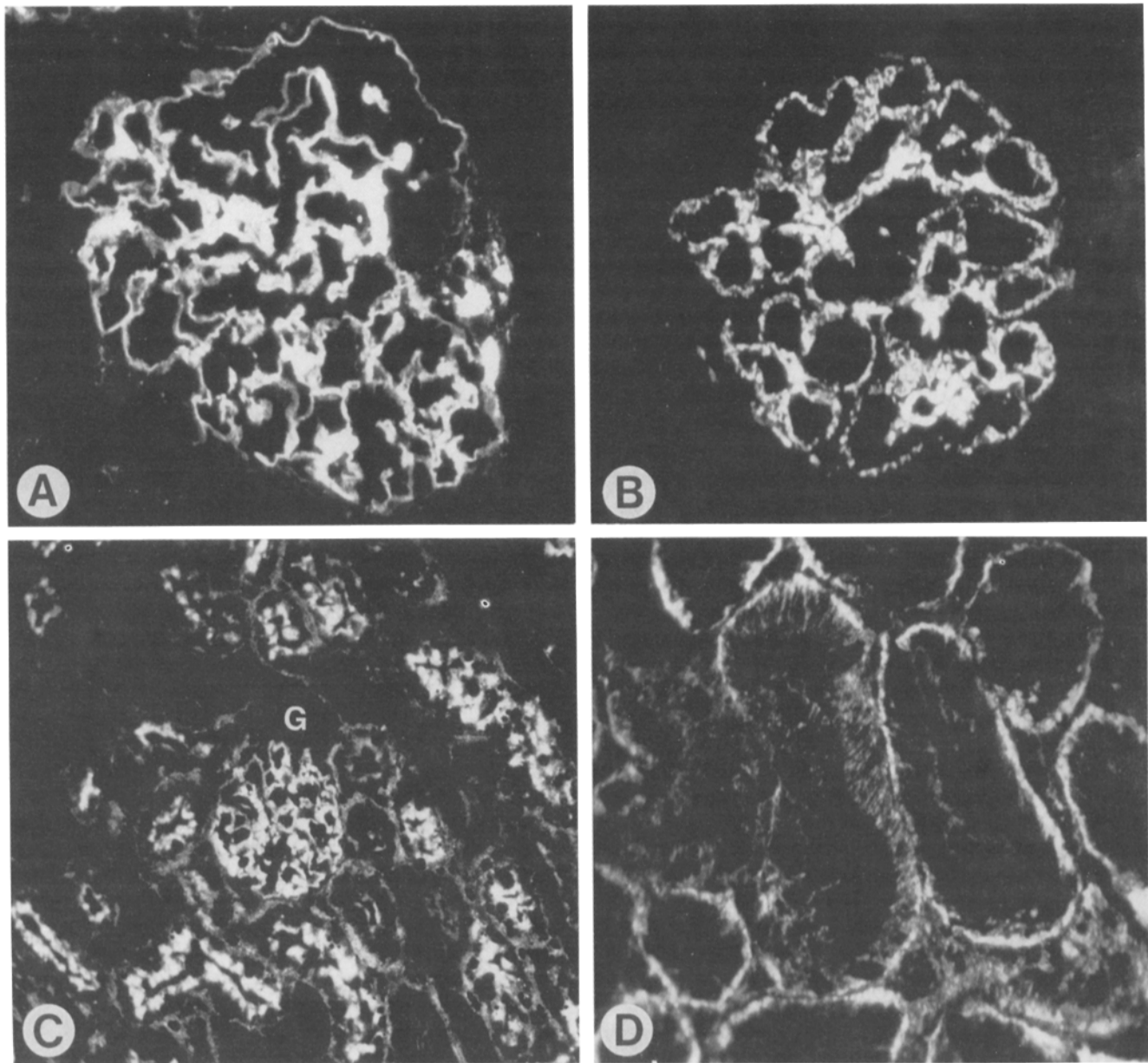


Figure 6. Direct immunofluorescence of kidneys of mice after intravenous administration of ASD-4. (A) Homogeneous binding to the glomerular capillary wall at 1 h ($\times 800$). (B) Fine granular binding in a membranous pattern at day 14 ($\times 800$). (C) Binding to the BB of the convoluted proximal tubules at day 1 (G, glomerulus) ($\times 320$). (D) Finely striped or more prominent binding to the basal parts of the convoluted proximal tubules at day 8. Binding to the BB has disappeared at this time ($\times 640$).

now also complexed with mouse IgG and in a few glomeruli with traces of mouse C3 (Fig. 6 B). The glomerular binding was accompanied by a transient binding to the BB of the convoluted proximal tubules during the first days after injection, followed by a strong binding to the basolateral parts of the convoluted proximal tubulus (Fig. 6, C and D). The binding to the BB was already present at 1 h after injection of the antibody (Table 4). During the first 8 d after injection, the mAb was also found on endothelial cells of the peritubular capillaries. 2 wk after injection, the mAb could only be observed in the glomerular capillary wall and to a lesser extent on the muscle cells of small arteries.

The distribution of the injected mAb could be examined more precisely by direct IEM using the immunoperoxidase labeling technique. 1 h after injection, ASD-4 was present particularly on the basal cell membranes and slit pores of the foot processes of the glomerular visceral epithelial cells, and sometimes also on the urinary side of some foot processes (Fig. 7 A). After 1 d, binding of ASD-4 to the cell membranes of the epithelial cells became gradually more variable and restricted to the slit pores, while in addition fine granular deposits appeared under the foot processes and in the slit pores. At day 14, the mAb could only be detected on the cell membranes of the epithelial cells near the slit pores or as deposits

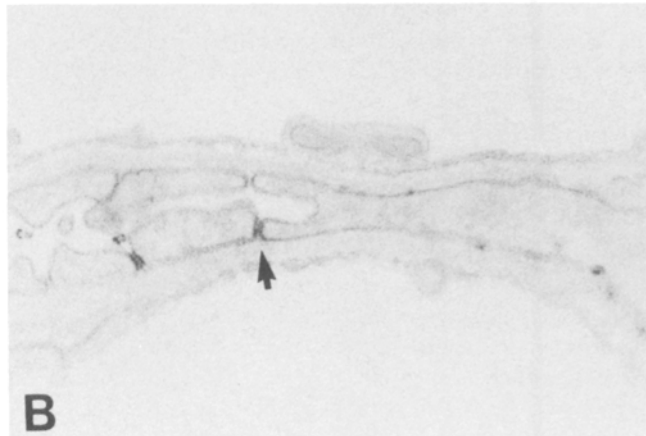
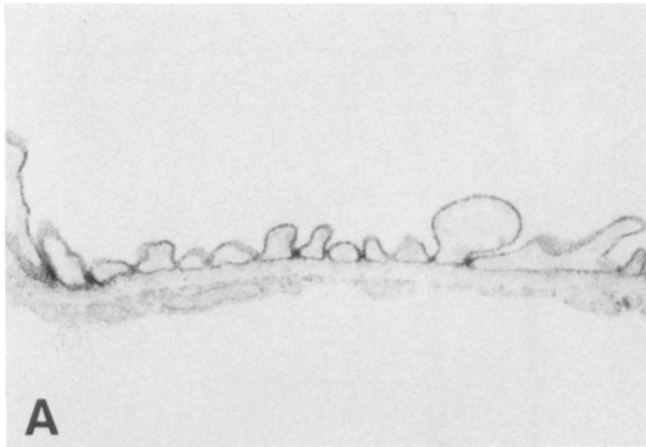


Figure 7. Direct immuno-electron microscopy of kidneys of mice after intravenous injection of ASD-4. (A) Binding is seen in the slit pores and on the cell membranes of the glomerular visceral epithelial cells at 1 h ($\times 16,530$). (B) At day 14, ASD-4 is present on the cell membranes of the foot processes along the slit pores (arrow) and in small deposits below the epithelial cells ($\times 19,600$).

in the slit pores and below the foot processes (Fig. 7 B). Binding of ASD-4 was always restricted to the epithelial side of the GBM. The GBM itself, mesangium, and endothelial cells did not show binding during the time of observation. ASD-4 could also be detected on the BB and subsequently in the basolateral parts of the convoluted proximal tubules, as well as on the endothelia of the peritubular capillaries as already described for IF.

Morphologic Changes after Injection of ASD-4. By LM, no glomerular lesions could be observed during the first 2 wk after injection of the mAb, notably no influx of polymorphonuclear leukocytes (PMN), platelets, or mononuclear cells could be seen. However, the cells of the convoluted and straight parts of the proximal tubulus became gradually more finely vacuolated. At the ultrastructural level at day 1, partial fusion of the foot processes of the visceral epithelia could be observed, which was almost complete 14 d after the injection of the mAb (Fig. 8). No abnormalities of the endothelia or mesangium could be seen. Only at day 14 were a few widely

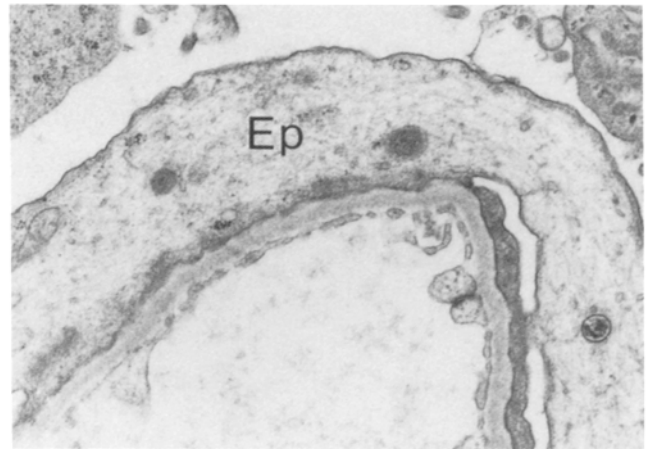


Figure 8. Ultrastructural findings 14 d after injection of ASD-4. A. Complete effacement of the foot processes of the visceral epithelial cells is present ($\times 18,400$). Ep, epithelial cell.

scattered electron-dense immune deposits visible in the slit pores of the visceral epithelia.

Nephritogenic Properties and Effects on APA Activity of Other mAbs to APA. Injection of two other mAbs to APA (ASD-2 and ASD-3) caused a massive albuminuria at day 1 (Table 1). Like ASD-4, they could reduce the APA activity on BB vesicles by $\sim 90\%$. Five other mAbs to APA were unable to induce enhanced glomerular permeability at day 1 with albumin excretions remaining not different from the values obtained with 0.7 ml of PBS alone or with the two control mAbs. Two of these mAbs, ASD-37 and ASD-39, also inhibited the enzymatic activity of the BB vesicles considerably, while the three remaining mAbs, ASD-38, ASD-41, and ASD-44, had no inhibitory effect on APA activity (Table 1). All mAbs that had similar, high binding titers in the indirect IF on normal mouse kidneys showed comparable glomerular binding 1 d after injection into the mice.

The control mAbs ASD-17 and ASD-43 did not induce albuminuria at day 1, despite a strong binding to the glomerular capillary wall as seen by direct IF. They were also not able to influence the APA activity on BB vesicles.

Discussion

Proteinuria is an important hallmark of glomerular disease, and several mechanisms that mediate enhanced glomerular permeability have now been delineated in a variety of experimental models (reviewed in references 1 and 3). In this report we describe a rat mAb that showed unique proteinuric properties after a single intravenous injection into normal mice. The albuminuria was dose dependent and lasted for at least 16 d. Its induction did not seem to be related to any of the known systemic mediators of glomerular injury, such as complement, coagulation factors, monocytes, PMN, or platelets. A rapid, homogeneous binding to the cell membranes of the glomerular visceral epithelial cells was observed, which very soon became finely granular with a preferred lo-

calization in the slit pores of the epithelial cell foot processes. These subepithelial deposits were accompanied by an effacement of the foot processes of the glomerular visceral epithelial cells. There was no cell detachment as seen in some models of experimental glomerulonephritis.

Besides the immediate binding to the glomerular capillary wall, a transient binding of ASD-4 to the BB of the convoluted proximal tubules, obviously due to the massive leakage through the glomerular filter, was seen during the first 2 d after injection of the mAb. Staining of the BB was seen as early as 1 h after injection, suggesting that the protein leakage started almost immediately. This was followed by a binding to the basolateral membranes of these cells, which had disappeared at day 14. The time sequence of binding of ASD-4 to the different parts of the convoluted proximal epithelial cells suggests an active transport of the mAb through the cytoplasm to the basolateral membranes after binding to the BB. An alternative explanation might be that ASD-4 can bind directly to the basolateral membranes via the peritubular capillaries after an upregulation of APA at these membranes, which show minute amounts of enzyme in normal, physiological circumstances as shown by indirect IF and IEM.

The evidence that ASD-4 is directed against the hydrolase APA (angiotensinase A) was based on several data. (a) ASD-4 bound in the immunoprecipitation procedure to an antigen with a molecular mass identical to that reported for APA isolated from murine pre-B cells, i.e., 140 kD (34). (b) ASD-4 could deplete APA activity from a mouse BB preparation without affecting the activities for APN and DPP IV. (c) A mild elution procedure with a basic buffer could specifically recover nearly 40% of the activity for APA from Sepharose-4B beads used in the immunoabsorption procedure. (d) The presence of APA in the mouse kidney corresponds to the localization of APA as previously assessed by histochemical studies (35). From these histochemical studies it is already known that the two hydrolases, DPP IV and APA, have almost identical localizations in the mouse and rat kidney (7, 35–41). Both proteins are prominently present on the cell membranes of the glomerular visceral epithelial cells, on BB of the proximal tubular cells, and on endothelial cells. There are, however, several differences: APA is absent from the glomerular endothelial cells and only moderately present on the straight parts of the proximal tubules in contrast to DPP IV, which is located on both structures. In addition, APA is also present in juxtaglomerular cells and on cell membranes of smooth muscle cells of arteries. In all these respects ASD-4 localized in our study in a pattern similar to that of APA. Besides differences in localization, the two hydrolases, DPP IV and APA, show different molecular masses and different reactions after binding with their respective mAb. In preliminary studies with several rat mAbs against mouse DPP IV produced in our laboratory, we found an immediate homogeneous binding to the glomerular capillary wall that showed no redistribution during 8 d after intravenous injection. Furthermore, we could not induce albuminuria, even when doses up to 15 mg of mAb were injected. Polyclonal antibodies against mouse DPP IV caused a minor, transient albuminuria,

which was only a fraction of the albuminuria induced by ASD-4 (11).

Recently, the cDNA sequence of APA purified from an Abelson murine leukemia virus-transformed murine pre-B cell line was elucidated (42). APA, formerly known as the murine B lymphocyte antigen BP-1/6C3 present on normal and transformed pre-B and immature B lymphocytes, proved to be a homodimeric, phosphorylated cell surface glycoprotein with subunits of 140 kD present on a variety of hematopoietic and epithelial cells (39). The cDNA sequence determined a type II integral membrane protein of 945 amino acids with an intracytoplasmic NH₂ terminus of only 17 amino acids and a typical zinc-binding motif in the extracellular domain. Despite this, APA is a zinc-independent enzyme that needs Ca²⁺ at its active site as a necessary requirement for its full activity. Although most APA are cell membrane bound, a soluble form can also be detected in the serum, generated most likely by proteolytic cleavage from the membrane form.

The functions of APA present on cell membranes of many cell types, together with several ectoenzymes, are varied and are related to digestion and absorption of peptides in the renal tubules or to a regulatory role in several regulatory systems on cells. APA has a well-known function in the renin-angiotensin system by cleaving the NH₂-terminal amino acid aspartate from the angiotensin II and to a lesser extent also from angiotensin I (34, 38, 39). It is one of the so-called angiotensinases involved in the rapid inactivation of the angiotensins. The octapeptide angiotensin II is the most active pressor substance of the renin-angiotensin system, which controls systemically or locally many functions in nearly every organ (43–46). With respect to the kidney, it is particularly involved in the control of renal and glomerular blood flow, the glomerular filtration rate, and the absorption of sodium ions by tubular cells (47). In addition to these hemodynamic and homeostatic functions, angiotensin II has also mitogenic properties and may thus have a function as a growth factor (48). The localization of APA in the kidney corresponds roughly to the localization of the receptors for angiotensin II and suggests a regulatory role of APA in the different functions of angiotensin II. Receptors for angiotensin II have been demonstrated in the glomeruli, particularly on mesangial cells, on smooth muscle cells of vessels, on vasa recta bundles in the renal medulla, and on proximal tubular cells (49–51). In the glomerulus, binding of angiotensin II to its receptor on mesangial cells induces contraction of these cells influencing the glomerular capillary surface area available for filtration and consequently the glomerular capillary ultrafiltration coefficient (K_f) (51). At this moment, it is thought that APA on glomerular epithelial cells may bind and rapidly inactivate angiotensin II delivered to the glomerulus and then filtered via the capillary wall (52, 53).

It is not clear how the binding of the mAb to APA on the glomerular visceral epithelial cells can induce an albuminuria in mice. At least two possible mechanisms may be operating in this model during the first 16 d after injection of the mAb that act either alone or synergistically. The first mechanism may be hemodynamically determined by the

inhibitory effect of the injected mAb on the binding of angiotensin II to APA and its subsequent inactivation, leading to a prolonged and enhanced effect of this pressure peptide on the glomerular circulation and the traffic of proteins across the glomerular capillary wall. In several experimental conditions, it has been shown that exogenous as well as endogenous angiotensin II can increase the excretion of proteins in rats by hemodynamic factors (54, 55). The proteinuria described in these studies, however, is of short duration, most probably due to the rapid inactivation of angiotensin II. Although the results of the three nephritogenic mAbs that can inhibit the APA activity on BB vesicles are in line with this mechanism, the data obtained with two of the five non-nephritogenic mAbs that also could reduce enzymatic activity do not support the hypothesis that blocking of the active site on APA alone is responsible for the induction of the albuminuria.

Therefore, a second mechanism, probably related to a direct disturbance of the glomerular filter by the deposited ASD-4, might be more important in this model. There are several reports in which heterologous polyclonal antibodies, directed against antigens on glomerular visceral epithelial cells and mostly of noncomplementing-fixing subclass, are able to induce proteinuria without the activation of complement, coagulation factors, or the influx of inflammatory cells (reviewed in reference 3). The mechanism by which these antibodies cause proteinuria is not clear, but it has been proposed that the binding of mAb to cell membrane-associated proteins causes the release from the epithelial cell, of cytokines, or other as yet undefined compounds that induce physicochemical alterations in the GBM leading to enhanced permeability (3).

Each of these supposed mechanisms could be responsible for the acute occurrence of albuminuria after the injection

of ASD-4. It is likely that autologous antibodies, which became bound to the primary deposits of heterologous rat antibodies at later stages, and which apparently did not lead to activation and deposition of complement, contributed to the persistence of the proteinuria.

In contrast to the studies with polyclonal antibodies, most attempts to induce a proteinuria with single injections of mAbs directed against glomerular visceral epithelial cells have failed. In recent years, two mAbs have been described that could induce a complement- and cell-independent proteinuria in rats (17, 21). The first mAb against a 115/107-kD protein on glomerular visceral epithelial cells evoked a transient proteinuria lasting 6–8 d, but only when given in excessive amounts (66 mg of mAb) to rats pretreated with CFA (17). The second mAb against a 56-kD protein present on the slit diaphragms of the glomerular visceral epithelial cells caused a proteinuria that lasted for ~2 wk (21).

In addition to its ability to induce a reproducible and persistent proteinuria in mice, ASD-4 has the advantage of being directed against a molecule of which the localization and the functions are well known. It is important to note that not every antibody against APA will cause albuminuria. Apparently the antibody must be directed against specific epitopes on the APA molecule, since we found that some of the other antibodies against APA, prepared in a similar way by us, did not induce proteinuria, although their *in vitro* and *in vivo* binding pattern was identical to that of ASD-4.

The antibody described here provides an opportunity to study the pathogenic mechanisms of proteinuria in a model in which the well-known secondary mediator systems do not appear to play a role. Studies in which we try to elucidate the role of angiotensin II inactivation by ASD-4 on the glomerular circulation and the ensuing albuminuria are now under way in our laboratory.

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References

1. Couser, W.G. 1985. Mechanisms of glomerular injury in immune-complex disease. *Kidney Int.* 28:569.
2. Brentjens, J.R., and G. Andres. 1989. Interaction of antibodies with renal cell surface antigens. *Kidney Int.* 35:954.
3. Couser, W.G. 1990. Mediation of immune glomerular injury. *J. Am. Soc. Nephrol.* 1:13.
4. Kerjaschki, D., and M.G. Farquhar. 1982. The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. *Proc. Natl. Acad. Sci. USA.* 79:5557.
5. Kerjaschki, D., and M.G. Farquhar. 1983. Immunocytochemical localization of the Heymann nephritis antigen (gp330) in glomerular epithelial cells of normal Lewis rats. *J. Exp. Med.* 157:667.

6. Salant, D.J., R.J. Quigg, and A.V. Cybulsky. 1989. Heymann nephritis: mechanisms of renal injury. *Kidney Int.* 35:976.
7. Ronco, P., L. Allegri, O. Melcion, E. Pirotsky, M.D. Appay, J. Bariety, F. Poutillon, and P. Verroust. 1984. A monoclonal antibody to brush border and passive Heymann nephritis. *Clin. Exp. Immunol.* 55:319.
8. Ronco, P., C. Melcion, M. Geniteau, E. Ronco, L. Reininger, M. Galceran, and P. Verroust. 1984. Production and characterization of monoclonal antibodies against rat brush border antigens of the proximal convoluted tubule. *Immunology.* 53:95.
9. Natori, Y., I. Hayakawa, and S. Shibata. 1986. Passive Heymann nephritis with acute and severe proteinuria induced by heterologous antibody against renal tubular brush border glycoprotein gp 108. *Lab. Invest.* 55:63.
10. Verroust, P., P. Ronco, and F. Chatelet. 1987. Antigenic targets in membranous glomerulonephritis. *Springer Semin. Immunopathol.* 9:341.
11. Assmann, K.J.M., M.M. Tangelder, W.P.J. Lange, Th.M. Tadema, and R.A.P. Koene. 1983. Membranous glomerulonephritis in the mouse. *Kidney Int.* 24:303.
12. Assmann, K.J.M., P. Ronco, M.M. Tangelder, W.P.H. Lange, P. Verroust, and R.A.P. Koene. 1985. Comparison of the antigenic targets involved in antibody-mediated glomerulonephritis in the mouse and the rat. *Am. J. Pathol.* 121:112.
13. Assmann, K.J.M., P. Ronco, M.M. Tangelder, W.P.H. Lange, P. Verroust, and R.A.P. Koene. 1989. Involvement of an antigen distinct from the Heymann antigen in membranous glomerulonephritis in the mouse. *Lab. Invest.* 60:138.
14. Kerjaschki, D., D.J. Sharkey, and M.G. Farquhar. 1984. Identification and characterization of podocalyxin: The major sialoprotein of the renal glomerular epithelial cell. *J. Cell Biol.* 98:1591.
15. Huang, T.W., and J.C. Langlois. 1985. A new cell surface protein of the podocyte and endothelium. *J. Exp. Med.* 162:245.
16. Tauc, M., F. Chatelet, P. Verroust, A. Vandewalle, P. Poujeol, and P. Ronco. 1988. Characterization of monoclonal antibodies specific for rabbit renal brush-border hydrolases: application to immunohistological localization. *J. Histochem. Cytochem.* 36:523.
17. Mendrick, D.L., and H.G. Rennke. 1988. I. Induction of proteinuria in the rat by a monoclonal antibody against SGP-115/107. *Kidney Int.* 33:818.
18. Ozaki, I., I. Ito, A. Fukatsu, N. Suzuki, F. Yoshida, Y. Watanabe, N. Sakamoto, and S. Matsuo. 1990. A plasma membrane antigen of rat glomerular epithelial cell. Antigenic determinants involving N-linked sugar residues in a 140-kilodalton sialo-glycoprotein of the podocytes. *Lab. Invest.* 63:707.
19. Nishikawa, K., A. Fukatsu, H. Tamai, N. Suzuki, Y. Ito, N. Sakamoto, and S. Matsuo. 1991. Formation of subepithelial dense deposits in rats induced by a monoclonal antibody against the glomerular cell surface antigen. *Clin. Exp. Immunol.* 83:143.
20. Fukatsu, A., Y. Yuzawa, L. Olson, J. Miller, M. Milgrom, M.J. Zmlauski-Tucker, J.B. van Liew, A. Campagnari, N. Niesen, J. Patel, T. Doi, L. Striker, G. Striker, F. Milgrom, J. Brentjens, and G. Andres. 1989. Interactions of antibodies with human glomerular epithelial cells. *Lab. Invest.* 61:389.
21. Orikasa, M., K. Matsui, T. Oite, and F. Shimizu. 1988. Massive proteinuria induced in rats by a single intravenous injection of a monoclonal antibody. *J. Immunol.* 141:807.
22. Miettinen, A., G. Dekan, and M.G. Farquhar. 1990. Monoclonal antibodies against membrane proteins of the rat glomerulus. *Am. J. Pathol.* 137:929.
23. Dekan, G., A. Miettinen, E. Schnabel, and M.G. Farquhar. 1990. Binding of monoclonal antibodies to endothelium, slit membranes, and epithelium after in vivo injection. *Am. J. Pathol.* 137:913.
24. Malathi, T., A. Preiser, P. Fairclough, P. Mallet, and R.K. Crane. 1979. A rapid method for the isolation of kidney brush border membranes. *Biochim. Biophys. Acta.* 554:259.
25. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604.
26. Lowry, O.H., N.J. Rosebrough, R.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
27. Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.)* 256:495.
28. Mancini, O., A.O. Carbonara, and J.R. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* 2:235.
29. Coombs, R.R.A., M.L. Scott, and M.P. Cranage. 1987. Assays using red cell-labeled antibodies. *J. Immunol. Methods.* 101:1.
30. Markwell, M.A.K. 1982. A new solid-state reagent to iodinate proteins. 1. Conditions for the efficient labeling of antiserum. *Anal. Biochem.* 125:427.
31. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.
32. Castillo, M.J., K. Nakajima, M. Zimmerman, and J.C. Powers. 1979. Sensitive substrates for human leukocyte and porcine pancreatic elastase: a study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. *Anal. Biochem.* 99:53.
33. Assmann, K.J.M., M.M. Tangelder, W.P.J. Lange, G. Schrijver, and R.A.P. Koene. 1985. Anti-GBM nephritis in the mouse: severe proteinuria in the heterologous phase. *Virchows Arch. Pathol. Anat. Histopathol.* 406:285.
34. Wu, Q., M.D. Cooper, M. Pierres, and J.P. Gorvel. 1991. Aminopeptidase A activity of the murine B-lymphocyte differentiation antigen BP-1/6C3. *Proc. Natl. Acad. Sci. USA.* 88:676.
35. Kugler, P. 1981. Localization of aminopeptidase A (Angiotensinase A) in the rat and mouse kidney. *Histochemistry.* 72:269.
36. Lojda, Z., and R. Gossrau. 1980. Study on aminopeptidase A. *Histochemistry.* 67:267.
37. Kugler, P. 1982. Ultracytochemistry of aminopeptidase A (angiotensinase A) in the kidney glomerulus and juxtaglomerular apparatus. *Histochemistry.* 74:199.
38. Kugler, P. 1982. Aminopeptidase A is angiotensinase A. I. Quantitative histochemical studies in the kidney glomerulus. *Histochemistry.* 74:229.
39. Kugler, P. 1982. Aminopeptidase A is angiotensinase A. II. Biochemical studies on aminopeptidase A and M in rat kidney homogenate. *Histochemistry.* 74:247.
40. Gossrau, R. 1979. Peptidases II. Zur lokalization der dipeptidylpeptidase IV (DPP IV). Histochemische und biochemische untersuchung. *Histochemistry.* 60:231.
41. Kenny, A.J., and S. Maroux. 1982. Topology of microvillar membrane hydrolases of kidney and intestine. *Physiol. Rev.* 62:92.
42. Wu, Q., J.M. Lahti, G.M. Air, P.D. Burrows, and M.D. Cooper. 1990. Molecular cloning of the murine BP-1/6C3 antigen: a member of the zinc-dependent metallopeptidase family. *Proc. Natl. Acad. Sci. USA.* 87:993.
43. Dzau, V.J. 1988. Circulating versus local renin-angiotensin system in cardiovascular homeostasis. *Circulation.* 77(Suppl. I):F4.

44. Dzau, V.J., D.W. Burt, and R.E. Pratt. 1988. Molecular biology of the renin-angiotensin system. *Am. J. Physiol.* 255:F563.
45. Dzau, V.J. 1989. Multiple pathways of angiotensin production in the blood vessel wall: evidence, possibilities, and hypotheses. *J. Hypertens.* 7:933.
46. Campbell, D.J. 1987. Circulating and tissue angiotensin systems. *J. Clin. Invest.* 79:1.
47. Navar, L.G., and L. Rosivall. 1984. Contribution of the renin-angiotensin system in the control of intrarenal hemodynamics. *Kidney Int.* 25:857.
48. Katz, A.M. 1990. Angiotensin II: hemodynamic regulator or growth factor. *J. Mol. Cell. Cardiol.* 22:739.
49. Mendelsohn, F.A.O., M. Dunbar, A. Allen, S.T. Chou, M.A. Millau, G. Aguilera, and K.J. Catt. 1986. Angiotensin II receptors in the kidney. *Fed. Proc.* 45:1420.
50. Douglas, J.G. 1987. Angiotensin receptor subtypes of the kidney cortex. *Am. J. Physiol.* 253:F1.
51. Yamada, H., P.M. Sexton, S.Y. Chai, W.R. Adam, and F.A.O. Mendelsohn. 1990. Angiotensin II receptors in the kidney. Localization and physiological significance. *Am. J. Hypertens.* 3:250.
52. Kugler, P., and T.H. Schiebler. 1984. Quantitative histochemistry of angiotensinase A (APA) in the renal glomeruli of rats after stimulation of the renin-angiotensin system. *Cytometry.* 5:392.
53. Wolf, G., J.E. Scherberich, G. Stuckhardt, and W. Schöppe. 1987. Characterization of angiotensin receptors from human kidney. *Protides Biol. Fluids Proc. Colloq.* 35:289.
54. Bohrer, M.P., W.M. Deen, C.R. Robertson, and B.M. Brenner. 1977. Mechanism of angiotensin II-induced proteinuria in the rat. *Am. J. Physiol.* 253:F13.
55. Yoshioka, T., T. Mitarai, V. Kon, W.M. Deen, H.G. Rennke, and I. Ichikawa. 1986. Role for angiotensin II in an overt functional proteinuria. *Kidney Int.* 30:538.