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Monoclonal antibodies to human glomerular antigens*

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Summary. Using cultured human fetal kidney cortical cells as antigen, two monoclonal antibodies (moAbs) against human glomeruli were produced. One of these moAbs, H-4, recognized the cell surface of glomerular epithelial cells, and the other, H-13, recognized the extracellular matrix present in the mesangial area. Both also reacted with liver, H-4 recognizing antigen present on the hepatocyte, and H-13 recognizing antigen distributed along the sinusoid. Species specificity for these moAbs was examined using mouse, rat, guinea pig and rabbit glomeruli, which revealed that H-4 reacted with rat glomerular epithelial cells and H-13 stained guinea pig glomerular mesangium. In the human fetal kidney, H-13 reacted with the mesangium, glomerular and tubular basement membrane and Bowman's capsule, and H-4 with the glomerular and tubular epithelial cells. Dot immunobinding assay of fibronectin purified from glomerular culture supernatant and plasma revealed that H-13 recognized both plasma and cellular fibronectin. Immunoblot analysis of 2.0 M guanidine HCl extract after dissociation in sodium dodecyl sulfate and electrophoresis demonstrated binding of H-4 to a 125 kd polypeptide. Immunoblot analysis of thermolysin-digested fibronectin exhibited binding of H-13 to 145 kd and 110 kd fragments, but not to 38 kd – 29 kd fragments. In renal biopsy specimens from patients with membranous nephropathy, H-13 stained the glomerular basement membrane (GBM), but not the mesangium, whereas anti-fibronectin antisera stained both the GBM and the mesangium. In those from patients with minimal change nephrotic syndrome (MCNS), IgA glomerulonephritis (IgAGN) and membranoproliferative glomerunephritis (MPGN), the staining pattern with H-13 was similar to that with polyclonal anti-fibronectin antisera. These results indicate that H-4 recognizes a 125 kd polypeptide constituent of the glomerular epithelial cell membrane and that H-13 recognizes the cell binding domain of fibronectin as well as revealing structural alterations in the mesangium and GBM.

Key words: Monoclonal antibodies – Glomerular epithelial antigen – 125 Kd polypeptide – Cell binding domain of fibronectin

Introduction

In human glomerular diseases, immune reactants such as immunoglobulins, complement, and coagulation factors are found in the glomeruli, suggesting that these factors play a role in the initiation of the glomerular disease. However, the mechanisms of the glomerular cell proliferation and alterations in the nature and distribution of extracellular matrices in the various glomerular diseases remain largely unknown.

The introduction of the hybridization technique (Kohler and Milstein 1975) has facilitated the production of monospecific antibodies against cell surface antigens and the domain of extracellular matrices. These antibodies can be used to identify the cell type and to analyse the structure and distribution of extracellular matrices. Recently, some investigators have reported monoclonal antibodies (moAbs) reacting with kidney antigens (Hancock and Atkins 1983; Mendrick et al. 1983; Michael et al. 1983; Muller and Muller 1983). These reseachers used isolated glomeruli, isolated

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GBM, kidney cortical homogenate, or cultured kidney cortical cells as antigens and produced moAbs against glomerular and tubular epithelial cells as well as extracellular matrices including type IV collagen and fibronectin. Besides these moAbs, specific markers detected with moAbs to glomerular components are required for analysing the various glomerular diseases.

For the purpose of producing moAbs against glomerular constituents such as mesangial cells (to which no moAbs have yet been produced), epithelial cells and extracellular matrices, we have employed cultured human kidney cortical cells as an antigen for the following reasons. Firstly, these cells were expected to provide cell-associated antigen rich fraction. Secondly, these fetal cells include those with the potential for differentiating into mesangial or epithelial cells and the epitopes present on the differentiated cells are already manifest. Thirdly, these cells were expected to contain antigenic components which may reappear or increase in proliferating cells or regenerating matrices after glomerular injury.

For this study, an immunoblotting technique was employed for identifying the molecules on which antigenic determinants are present. In addition, immunohistologic methods were used for analysing the distribution of determinants recognized by moAbs. Two moAbs against human glomeruli have been described: H-4 reacting with a 125 kd polypeptide constitutent of the glomerular epithelial cell membrane and H-13 reacting with the cell binding domain of fibronectin.

Materials and methods

Male BALB/c mice were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Neonatal kidneys were removed at autopsy. The renal capsule was excised and the renal cortex was dissected away from the medulla using a sterile technique. The cortical slices were divided into 1–2 mm cubes and submitted to enzymatic digestion at 37° C for 15 min using 0.125% trypsin solution (DIFCO, USA) in Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+}. Trypsin digested cortical fragments were cultured in plastic flasks (#3013, Falcon, USA) containing 5 ml of RPMI1640 medium (GIBCO, USA), supplemented with 20% decomplemented fetal bovine serum (FBS) (GIBCO, USA), and maintained at 37° C in an atmosphere of 95% air and 5% CO_2. Subcultures were performed using 5 mM ethylenediaminetetraacetic acid (EDTA) in Dulbecco's phosphate buffered saline.

Mice were injected subcutaneously with 1×10^5 cultured human fetal kidney cortical cells emulsified with Freund's complete adjuvant, followed by two intraperitoneal injections of 1×10^5 cells with a 2-week interval. Four days after the final immunization the mice were sacrificed and their spleens were removed for hybridization. The spleen cells were fused with NS/1 (P3-NS1/1-AG4-1) myeloma cells using polyethylene glycol 3000, as described previously (Shimizu et al. 1984). Beginning at 24 h, hybridomas were selected in an HAT medium (supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.1×10^{-5} M thymidine) for 10 days, and cultured in an HT medium (HAT medium without added aminopterin) for another 10 days, and then grown in an RPMI1640 medium containing 15% FBS. The culture supernatant was screened for the presence of antibodies by indirect immunofluorescence. Selected hybridomas were cloned by a limiting dilution without a feeder layer, using the spent medium as a conditioning medium (cultured supernatant from NS-1 cells at the growing phase).

Cryostat sections from the normal portions of three kidneys removed surgically (kindly provided by Dr. Y. Sakata) and fetal kidneys (gestational age 14 and 28 weeks) were reacted with a drop of the culture supernatant and incubated at 37° C for 30 min. They were washed in phosphate buffered saline (PBS) for 15 min, stained with fluorescein isothiocyanate (FITC) labelled anti-mouse immunoglobulins (Cappel, USA) at 37° C for 30 min, washed again with PBS, and then mounted in buffered glycerol. The NS-1 supernatant was used as a control. The classes of moAbs were determined using FITC-conjugated antisera to mouse IgG_1, IgG_2, IgA and IgM (Meloy, USA), respectively. Sections were observed with a fluorescence microscope equipped with epi-illuminator (Zeiss, West Germany), as previously described (Nakamura et al. 1986). Photographs were taken with Fujichrome film (ASA-100). Double staining using tetramethyl rhodamine isothiocyanate (TRITC)-labeled anti human GBM was performed according to the method described by Suzuki et al. (1983).

For electron microscopy, normal human kidney tissue was fixed in periodate-lysine-paraformaldehyde solution (McLean and Nakane 1974) for 3 h and rinsed successively, in 10%, 15%, and 20% sucrose-PBS for 4 h, embedded in OCT compounds (Miles Lab. In., USA), and quickly frozen at −70° C, as described previously (Kazama et al. 1985). Sections (10 μm thick) were cut in a cryostat, placed on albuminized glass slides and rinsed in PBS three times for 5 min each. They were incubated with 10% normal goat serum-PBS for 30 min at room temperature, then incubated overnight at 4° C with H-4 or NS-1 culture supernatant. After washing they were incubated overnight at 4° C with biotinylated goat anti mouse IgG (EY Lab. In., USA), followed by another washing, and then incubated 5 h at 4° C with avidin-peroxidase (EY Lab. In., USA). The sections were fixed with 1% glutaraldehyde-PBS for 10 min and then were subjected to the 3-3′ diaminobenzidine (DAB) procedure according to Graham and Karnovsky (1966) followed by postfixation in osmium-phosphate buffer. The sections were dehydrated in graded ethanols and embedded in epoxy resin. Ultrathin sections were observed without staining.

In order to solubilize glomerular antigens, the glomeruli were isolated by the method by Burlington and Cronkite (1973). Cortical slices were minced with a razor blade and pressed with a spatula through a stainless steel screen of 60 mesh. They were rinsed with 50 mM Tris HCl, pH 7.4, containing 1 mM EDTA and 1 mM phenylmethylsulfonylchloride (PMSF) through successive screens of 100 mesh and 200 mesh placed in series. Tissue consisting of glomeruli on the 200 mesh screen was suspended in distilled water containing protease inhibitors, stirred for 2 h and centrifuged at 1000 g for 30 min. The supernatant was re-centrifuged at 60000 g for 30 min in order to collect the glomerular cell membrane fraction. The pellet was suspended and incubated in 1.0 M NaCl containing 2000 units of DNase (Sigma St. Louis, MO, USA) for 2 h at room temperature. DNase digested materials were centrifuged at 1000 g for 10 min. The precipitated glomerular fraction was solubilized

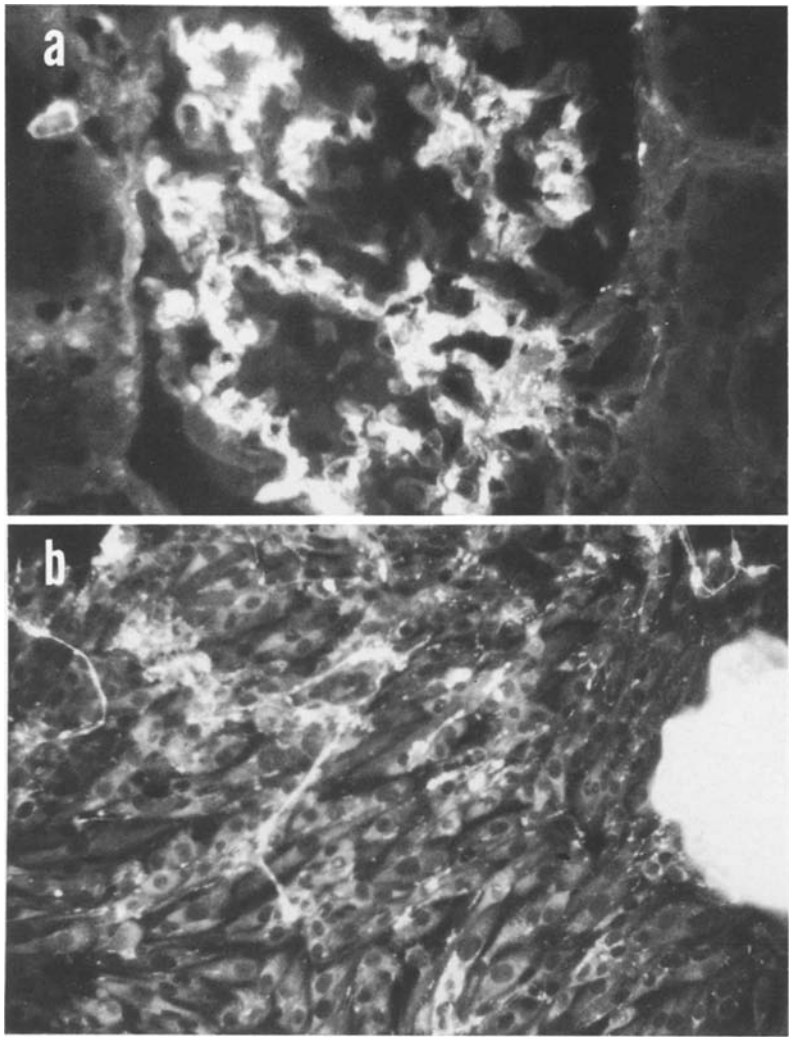

Fig. 1. Immunofluorescence microscopy of sections of human kidney and primary cultured glomeruli with H-13. **a**, H-13 reacts with the mesangium, but not with the GBM or interstitium (× 400). **b**, H-13 reacts with the extracellular matrix produced by the outgrowth cells in primary glomerular culture (× 200)

by 2.0 M guanidine HCl or 4.0 M urea, respectively. The glomerular cell membrane fraction was solubilized in 50 mM Tris HCl, pH 8.6, containing 1.0% deoxycholate (DOC) (DIFCO, USA).

Plasma fibronectin was purified on gelatin-sepharose 4B (Pharmacia, Sweden) from citrated fresh plasma according to Engvall and Ruoslahti (1977). Thermolysin (Sigma, USA) digestion of purified fibronectin was performed according to Sekiguchi et al. (1981). Purified fibronectin (1 mg/ml) in 25 mM Tris HCl, pH 7.6, containing 0.5 mM EDTA, 50 mM NaCl and 2.5 mM $CaCl_2$ was digested with 5 μg/ml of thermolysin for 4 h at 22° C. The digestion was terminated by adding EDTA (5 mM final concentration) to inactivate thermolysin. Collagen type IV was purchased from Sigma. Laminin was provided by Prof. M. Isemura (Shizuoka Women's Univ., Shizuoka).

The reactivity of moAbs to extracellular matrices and solubilized glomerular antigens was assayed by using Bio-Rad dot immunoassay apparatus, as previously described (Nakamura 1986). Samples (100 μl) were dispersed into wells and incubated for 30 min. After appropriate washing with 10 mM Tris-HCl, pH 7.4, containing 0.05% Tween 20, the sheet was blocked with 200 μl 1% solution of bovine serum albumin (BSA), and subsequently incubated with the culture supernatant of each well for 30 min. After washing, the sheet was incubated with peroxidase-labelled anti-mouse immunoglobulins (DAKO, Denmark). Peroxidase was detected by incubation with 4-chloro-1-naphtol and hydrogen peroxide.

The solubilized glomerular fraction and the thermolysin digested fibronectin were analysed by SDS-PAGE, as described by Laemmli (1970). Gradient polyacrylamide gels (5–15%) were used. The gels were stained with 0.25% Coomassie blue in 10% acetic acid/50% methanol and destained with 10% acetic acid. For immunoblot analysis, solubilized glomerular proteins and the thermolysin digested fibronectin separated by electrophoresis on SDS-PAGE were transfered electrophoretically from the gel onto nitrocellulose sheets (constant voltage 30 V, 12 h) as described by Towbins et al. (1979). After immersion in 5% bovine serum albumin in 0.2 M NaCl/50 mM Tris HCl, pH 7.4, the sheets were incubated with a hybridoma culture supernatant, or rabbit anti-fibronectin antisera (Cappel, USA) (overnight at 4° C) and subsequently with a peroxidase conjugated rabbit antisera against mouse immunoglobulins or goat antisera against rabbit immunoglobulins. Peroxidase was detected by incubation with 4-chloro-1-naphtol and hydrogen peroxide.

Ninety five renal biopsy specimens were examined in this study. The pathological diagnoses were established using routine light, immunofluorescent and electron microscopy. These renal specimens were from patients with the following histopathological diagnosis; MCNS 15, IgAGN 40, membranous nephropathy 30 and MPGN 10.

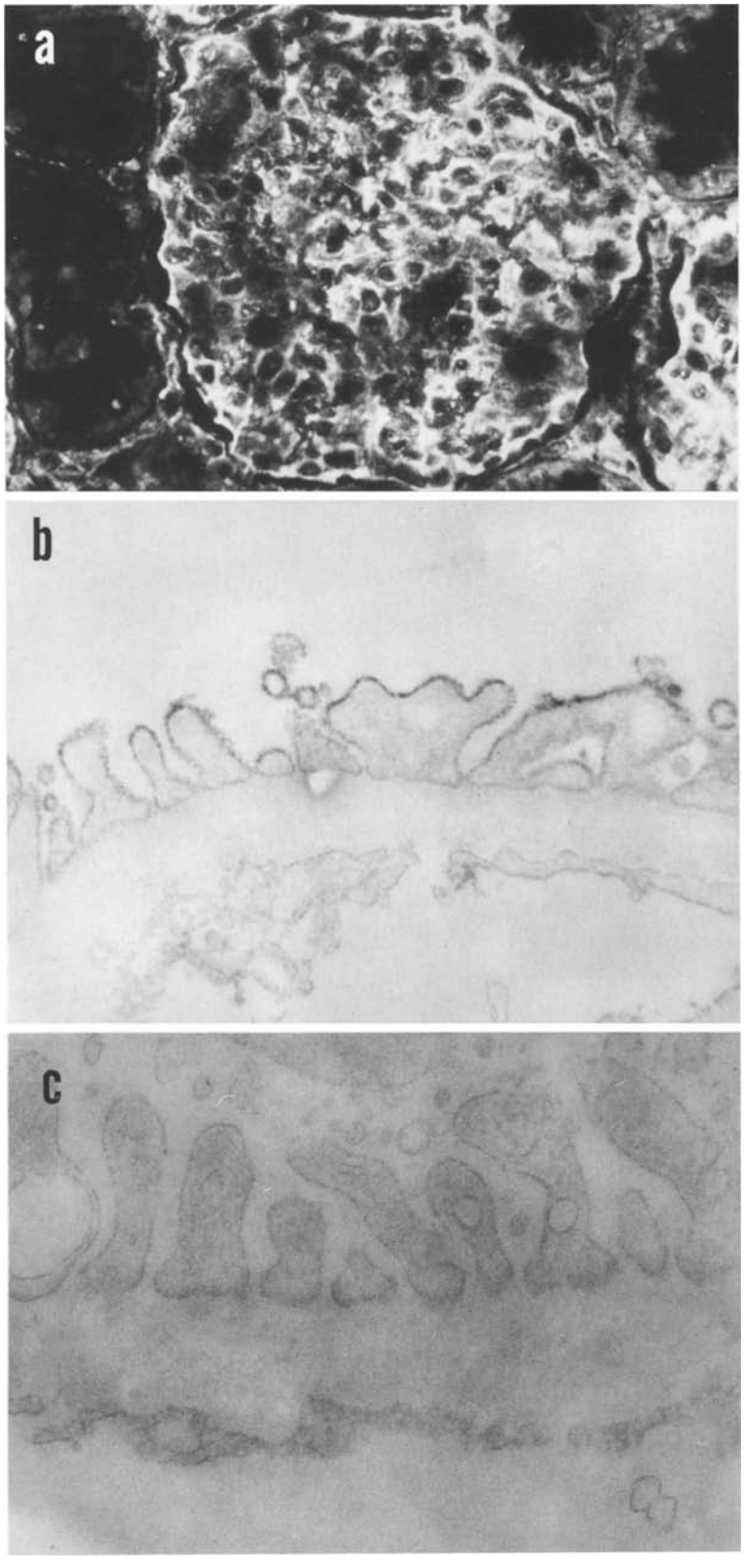

Fig. 2. Binding pattern of H-4 to glomerular structures. **a**, indirect immunofluorescence (×400). **b**, immunoelectron microscopy reveals reaction product exclusively on the surface of epithelial foot processes. The endothelial cell surface and the GBM do not bind this Ab (×50000). **c**, immunoelectron microscopy of NS-1 culture supernatant (×50000)

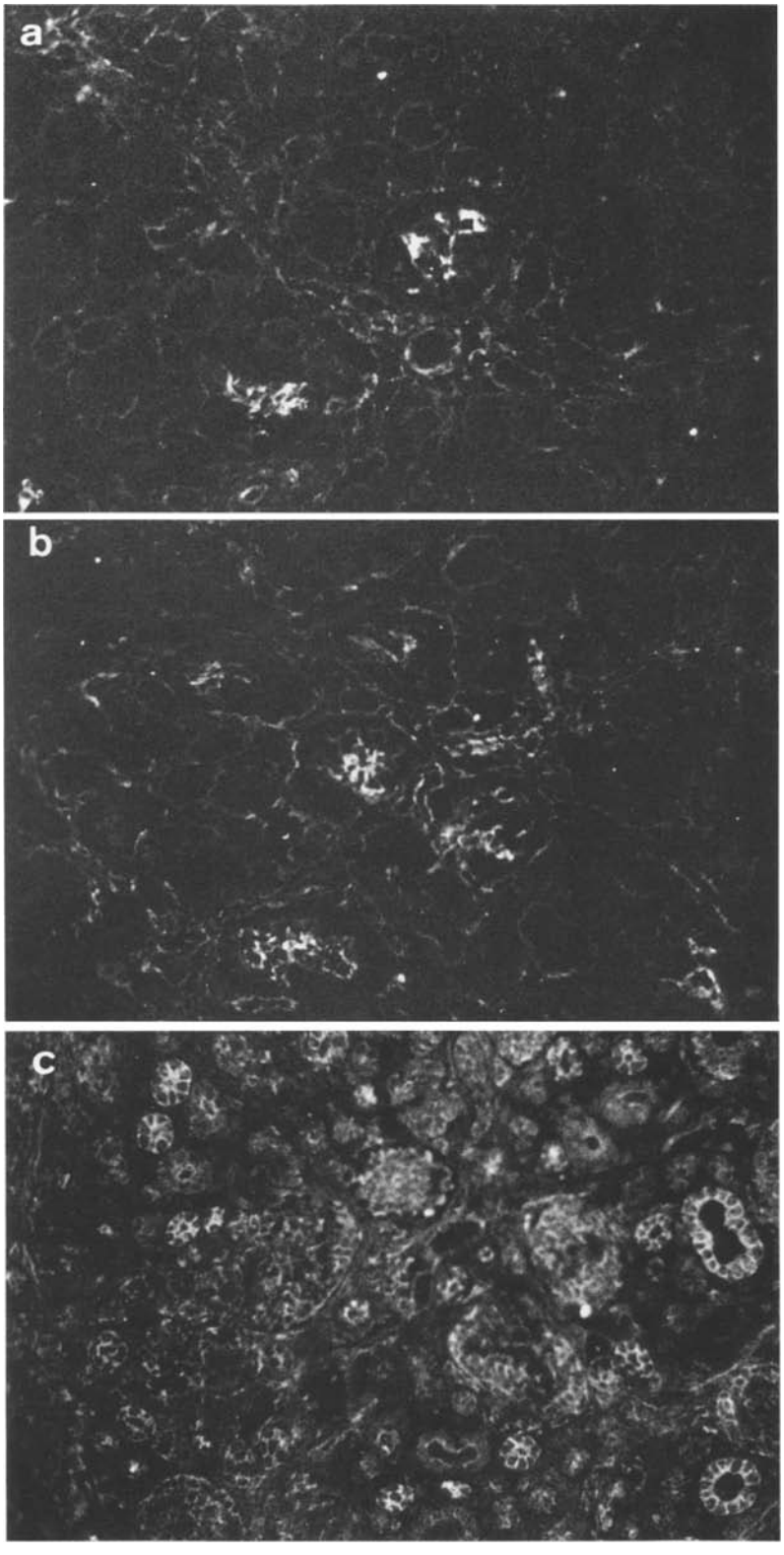

Fig. 3. Reactivity of moAbs with human fetal kidney. **a** and **b**, H-13 stains the mesangium, the GBM, the TBM and Bowman's capsule (gestational age; **a**, 14-week and **b**, 28-week) (×400). C, H-4 reacts with glomerular and tubular epithelial cells (gestational age; 28-week) (×400)

Table 1

	Col. (IV)	Lam.	FN (plasma)	FN (gl. cult.)	1.0% DOC	2.0 M G. HCl
H-4 (IgM)	(−)	(−)	(−)	(−)	(+)	(+)
H-13 (IgM)	(−)	(−)	(+)	(+)	(−)	(+)

1. Col. (IV); Type IV collagen.
2. Lam.; Laminin.
3. FN (plasma); fibronectin purified from plasma.
4. FN (gl. cult.); fibronectin purified from glomerular culture supernatant.
5. 1.0% DOC; 1.0% deoxycholate solubilized glomeruli.
6. 2.0 M G. HCl; 2.0 M guanidine HCl extracted glomeruli.

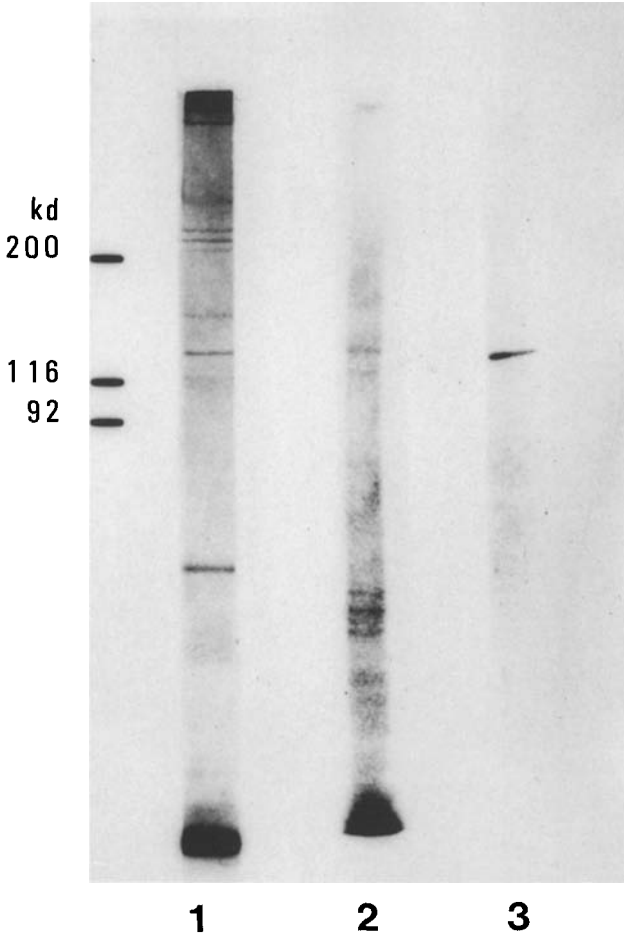

Fig. 4. 2.0 M guanidine HCl extracts of isolated glomeruli separated on 5–15% gradient SDS-gels (*lane 1*) and transferred onto nitrocellulose (*lane 2 and 3*). *Lane 1.* gel stained with Coomassie blue; *lane 2.* nitrocellulose sheet stained with Amido black; *lane 3.* nitrocellulose sheet incubated with H-4. The molecular mass markers are myosin (200,000), beta-galactosidase (116,250), phosphorylase b (92,500)

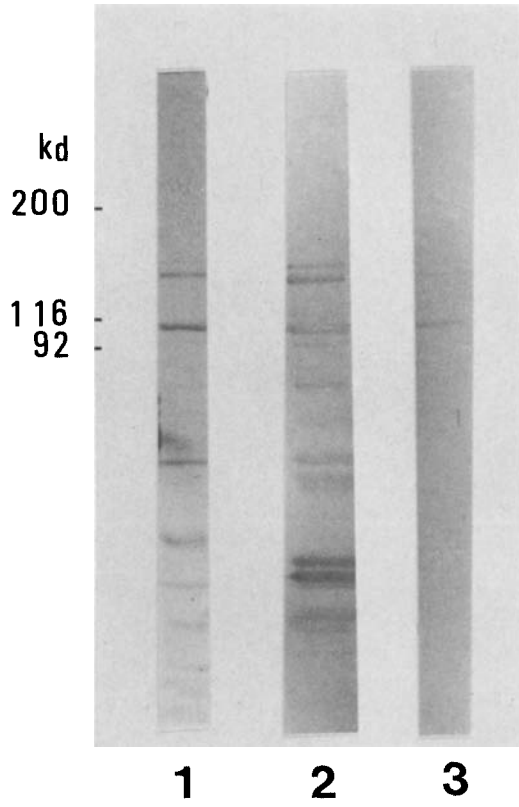

Fig. 5. Immunoblot analysis of thermolysin digested fibronectin. *lane 1.* nitrocellulose sheet stained with Amido black; *lane 2.* nitrocellulose incubated with anti fibronectin antisera; *lane 3.* nitrocellulose incubated with H-13. Molecular mass markers are described in the legend to Fig. 4

Results

Two moAbs (IgM class) were produced which reacted with renal glomeruli (Figs. 1a, 2a). H-13 stained the glomerular mesangium, but failed to stain the GBM or the renal interstitium (Fig. 1a). The fluorescence of H-4 appeared to be located along the epithelial surface of the GBM (Fig. 2a). This was confirmed by double staining using TRITC labeled anti-GBM antisera. Immunoelectron microscopic examination revealed that H-4 bound to the cell surface of glomerular epithelial cells (Fig. 2b). In fetal kidneys, H-13 stained the mesangium, GBM, TBM and Bowman's capsule (Fig. 3a, b), while H-4 reacted with glomerular and tubular epithelial cells (Fig. 3c).

H-4 reacted with the cell surface of the outgrowth cells in the primary glomerular culture. H-13 reacted with the extracellular matrix produced by the outgrowth cells in the primary glomerular culture (Fig. 1b).

H-4 stained rat glomeruli in the same pattern as with human glomeruli and H-13 reacted weakly with guinea pig glomerular mesangium.

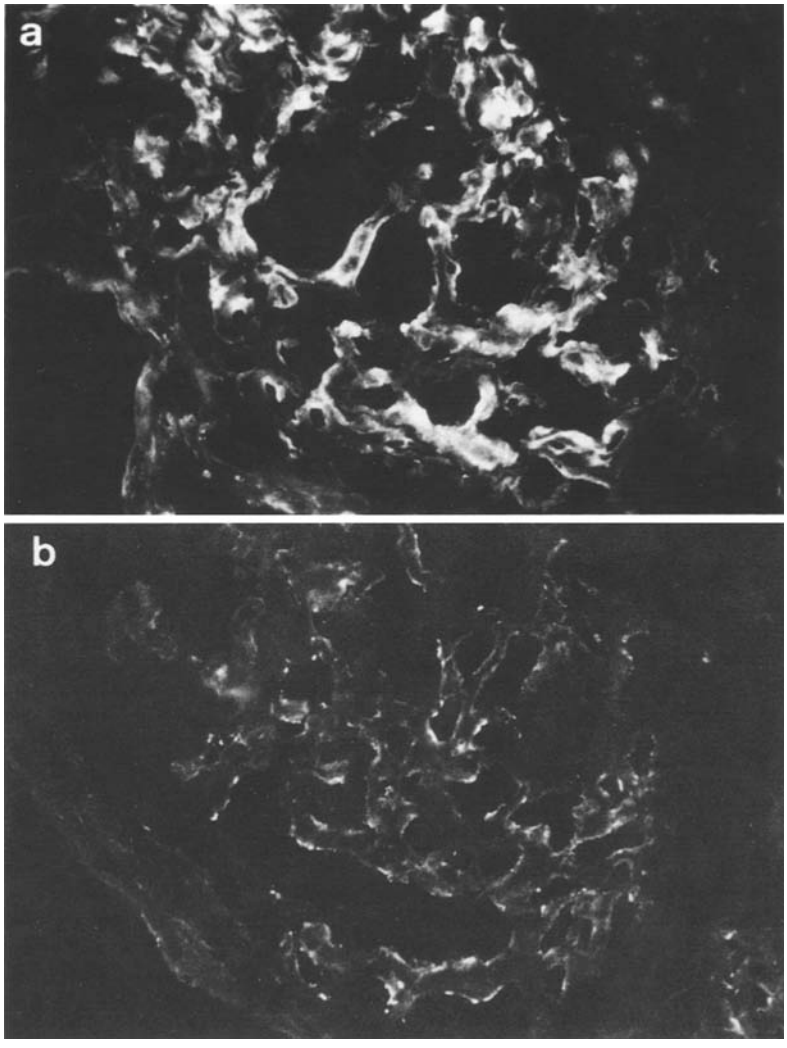

Fig. 6. Reactivity of H-13 and polyclonal anti-fibronectin antisera with the glomeruli of membranous nephropathy. **a**, Polyclonal anti-fibronectin antisera reacted with the mesangium and the GBM (×400). **b**, H-13 stained the GBM, but did not the mesangium (×400)

An antigen recognized by H-4 was present on the surface of hepatocyte, and an antigen recognized by H-13 was distributed along the sinusoids.

As shown in Table 1, H-4 reacted with the 2.0 M guanidine HCl extracted glomeruli and 1.0% DOC solubilized glomeruli, but failed to react with extracellular matrices such as fibronectin, laminin and collagen type IV. H-13 reacted with fibronectin purified from both plasma and glomerular culture supernatant and also with 2.0 M guanidine HCl extracts.

H-4 bound to 125 kd polypeptide in the 2.0 M guanidine HCl extract (Fig. 4). In the thermolysin digested fibronectin, H-13 reacted with 145 kd and 110 kd fragments, but failed to react with 38–29 kd fragments of fibronectin (Fig. 5).

In membranous nephropathy, H-13 reacted with the GBM, but not with the mesangium (Fig. 6b), whereas polyclonal anti-fibronectin antisera stained both the GBM and the mesangium (Fig. 6a). H-13 stained mainly the mesangium of glomeruli from patients with MCNS and IgAGN in the same pattern as polyclonal antisera did (Fig. 7a, b). In MPGN, H-13 stained both the GBM and the mesangium in the same pattern as polyclonal antisera di (Fig. 8a and b).

Discussion

In this study, we have described two moAbs against human glomeruli: H-4 recognized a 125 kd polypeptide constituent of the glomerular epithelial cell membrane, and H-13 recognized the cell binding domain of fibronectin. There have been a few previous reports describing moAbs against kidney antigens, characterized mainly by immunohistological methods. This approach proved to be effective not only in distribution analysis but also in the discrimination of antigenic determi-

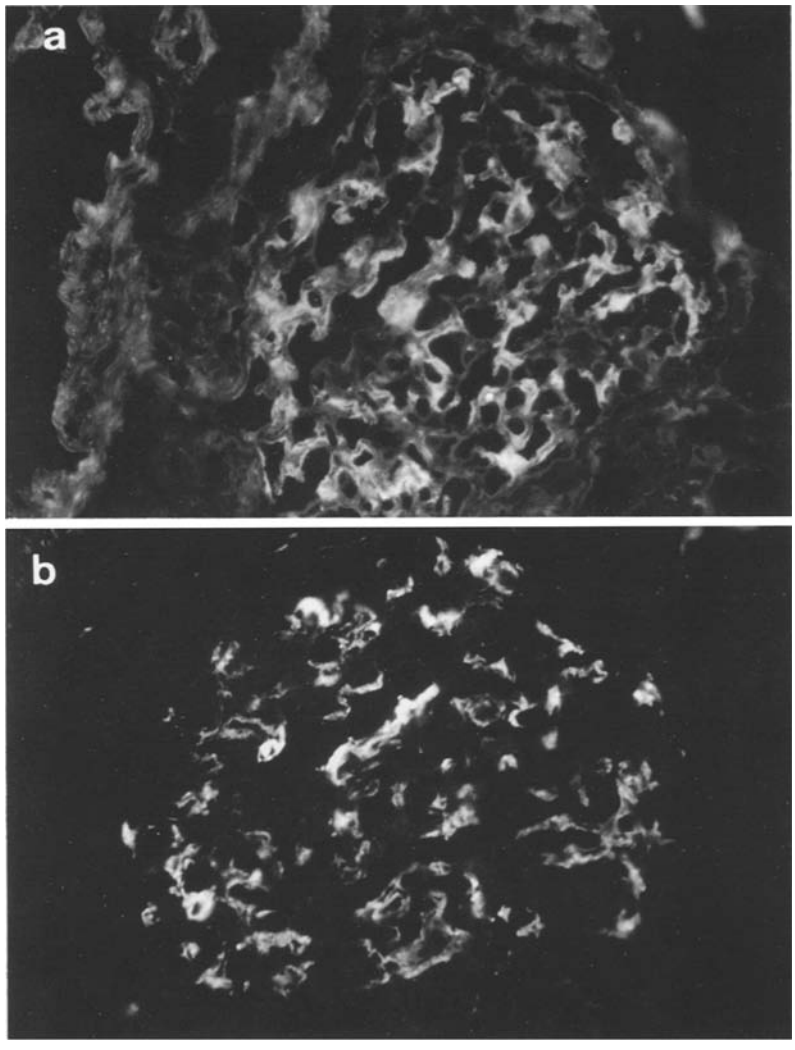

Fig. 7. Reactivity of H-13 and polyclonal anti-fibronectin antisera with the glomeruli of IgA GN. **a**, polyclonal antisera stained mainly the mesangium (×400). **b**, H-13 stained in the same pattern as polyclonal antisera (×400)

nants recognized by moAbs. Recently, moAbs against different determinants on the same molecules have been reported to show distinctly different patterns (Odermatt et al. 1984). It is therefore necessary to identify the molecules recognized by moAbs biochemically.

Fibronectin is found in many organs such as the kidney, intestine, lung, and placenta, and is also detected in plasma. Cellular fibronectin was initially reported as a cell surface component of cultured fibroblastic cells (Hynes 1976), and it has since been found on many different cell types (Yamada and Olden 1978; Vaheri and Mosher 1978; Mosher 1980). Fibronectin is also secreted by many cells including glomerular epithelial cells (Killen and Striker 1979), mesangial cells (Foidart et al. 1980), and has been found in culture media as a soluble protein (Hynes 1982). H-13 reacted with liver and uterine smooth muscle, but not with other organs including skin, lung, and placenta. Since the moAb to fibronectin which was described by Michael et al. (1983) reacted with liver, lung, and epidermis, this moAb did not seem to be directed against the determinant recognized by H-13 on fibronectin. Using indirect immunofluorescence, H-13 reacted with fetal kidney TBM and Bowman's capsule but not with these same structures in mature kidney. This loss of reactivity was also observed by Michael et al. (1983), and suggests that some components and/or the structure of the basement membrane may change during maturation. Immunoblot analysis of thermolysin digested fibronectin after dissociation in SDS and electrophoresis showed binding of H-13 to 145 kd and 110 kd fragments, but not to the 38 kd–29 kd fragment. Since the 145 kd fragment was reported to consist of cell binding and heparin binding domains, and the 110 kd fragment to be a cell binding

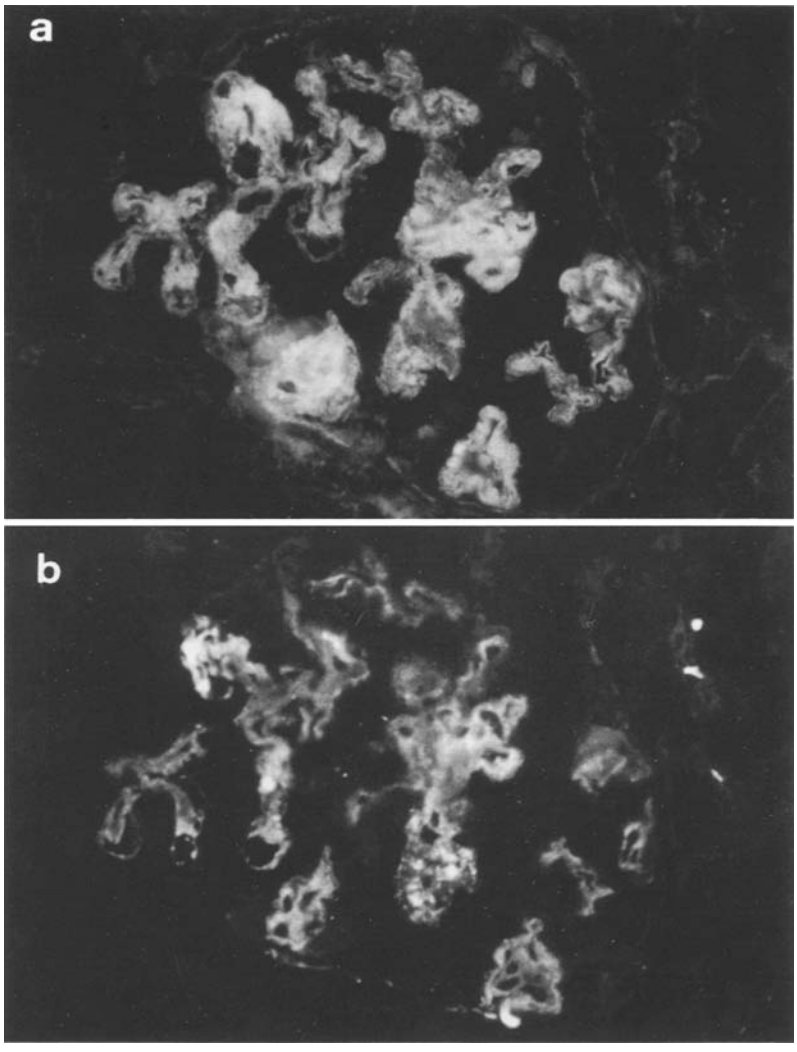

Fig. 8. Reactivity of H-13 and polyclonal anti-fibronectin antisera with the glomeruli of MPGN. **a**, polyclonal antisera stained both the mesangium and the GBM (×400). **b**, H-13 also stained both the mesangium and the GBM (×400)

domain (Zardi et al. 1985), H-13 recognized the cell binding domain of fibronectin.

Immunohistological examination revealed that determinant recognized by H-4 was expressed on the glomerular epithelial cell surface. Some moAbs against glomerular epithelial cells have been produced, one of them specific for human visceral epithelial cells (Muller and Muller 1983). Other moAbs against glomerular epithelial cells were shown to be species restricted (Hancock and Atkins 1983; Mendrick et al. 1983). H-4 reacted with human and rat glomerular visceral and parietal epithelial cells, indicating that H-4 is not directed against the same determinants recognized by these other moAbs. Glomerular epithelial cells express HLA antigens and C_3 receptors on their surface. HLA antigens consist of Class I and Class II polypeptides whose molecular weights are less than 100 kd. The C_3 receptor is a glycoprotein with a molecular weight of 205 kd. Since immunoblot analysis showed binding of H-4 to the 125 polypeptide, it does not appear to be directed against HLA antigens or C_3 receptors.

Three anionic sialoproteins have been isolated from the glomeruli by trypsin digestion, the molecular weights of which were 145 kd to 97 kd (Nevin and Michael 1981). Heterologous antisera against these proteins reacted with the cell surface and cytoplasm of epithelial cells and focally with endothelial cells. Recently, 140 kd protein has been reported to be a main sialoprotein of the glomerular epithelial cell, and to be localized on the surface of both glomerular epithelial cells and endothelial cells (Kerjaschki et al. 1984). While these heterologous antisera exhibited a staining pattern that was fundamentally different from H-4, the molecular weight of the antigen recognized by H-4 seems to be very similar to that of these sialopro-

teins. These findings suggest the possibility that H-4 recognized a sialoprotein associating determinant localized on the epithelial cell membrane. Isolation and characterization of the H-4 recognizing antigen is now in progress to investigate this possibility.

In summary, this study describes two moAbs against human glomeruli. The first, H-4, recognized a 125 kd polypeptide on the glomerular epithelial cell membrane, and the second, H-13, recognized the cell binding domain of fibronectin. H-4 may become a useful tool for analysing developmental changes in the antigenicity of renal epithelial cells with maturation. The differences in the staining pattern between H-13 and polyclonal anti-fibronectin antisera observed in membranous nephropathy reflects structural changes in fibronectin associated with alterations in both the GBM and the mesangium.

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