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Production and immunohistochemical characterization of monoclonal antibodies directed against renal basement membranes of rats

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Summary. Basement membranes were separated from rat glomeruli and purified by mild procedures, which led to a highly enriched basement membrane fraction.

Here, the production and characterization of five monoclonal antibodies against tubular and glomerular basement membranes are described. These antibodies were analyzed immunohistochemically on frozen sections of rat, bovine, and human kidneys as well as on rat embryos.

One monoclonal antibody (BM O II) exclusively recognized the glomerular basement membranes, another one (BM O VII) bound to tubular basement membranes and to Bowman's capsule. Three antibodies (BM O IV, BM M II, BM M III) recognized their antigens in both glomerular and tubular basement membranes as well as in mesangial cells. The BM O II antibody showed a stringent species specificity and bound only to glomerular basement membranes of the rat. The other four antibodies cross-reacted with human and bovine glomerular basement membrane and mesangial antigens; they also bound to other tissues in the developing rat embryo. Antibody binding to specific purified components of the basement membranes such as collagen type IV, laminin, heparan sulphate proteoglycan, and fibronectin was investigated by enzyme-linked immunosorbent assay (ELISA). None of these antibodies reacted with any of these known basement membrane components, indicating that the antibodies may serve as useful tools in future investigations of so far unidentified components of basement membranes.

Key words: Monoclonal antibodies, Glomerular basement membrane, Tubular basement membrane, Rat kidney

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Introduction

The basement membrane is the continuous barrier separating epithelium, endothelium, muscle cells and Schwann's cells from mesenchyme. Several disorders are associated with morphological and functional changes of basement membranes (Martínez et al., 1983). Basement membrane functions are suggested to involve transport and filtration in kidney, cellular adhesion, formation and maintenance of tissue integrity and functions in embryonic development and differentiation of tissues (Grant et al., 1981). Because of these different functions, basement membranes may contain different and unique components. Distinct morphological differences between basement membranes have been described (Hessle et al., 1984).

Although the identification of different components is difficult, because basement membranes cannot easily be solubilized, great efforts have been made in studying basement membranes morphologically and biochemically. Known basement membrane components are type IV collagen (Kephalides, 1973; Orkin et al., 1977), the glycoproteins, laminin (Timpl et al., 1979; Chung et al., 1976), entactin (Bender et al., 1981), nidogen (Timpl et al., 1983) and heparan sulfate proteoglycans (Kanwar and Farquhar, 1978; Hassel et al., 1980).

In studies of basement membrane components, monoclonal antibodies may be a valuable tool as a highly specific reagent for immunohistochemical studies. In recent years, monoclonal antibodies were produced against rat glomerular antigens (Mendrik et al., 1983) and human glomerular basement membranes (Falkenberg et al., 1981; Michael et al., 1983; Mino et al., 1984). Monoclonal antibodies against purified components of basement membranes, such as laminin (Wan et al., 1984), type IV collagen (Sundarraj et al., 1982; Odermatt et al., 1984) were also developed.

We separated glomerular basement membranes using a method developed by v. Bruchhausen and Merker (1964). This method is mild in comparison to other

methods described, where, for example, sonification, detergents, collagenase, and citrate extraction were used.

The aim of the present study was to identify and characterize new glomerular basement membrane components, which are normally lost by more or less crude methods of preparing and isolating basement membranes.

Materials and methods

Preparation of glomerular basement membranes

The kidneys of 100 Wistar rats were removed, collected in Ringer phosphate medium and passed through a steel sieve with a pore diameter of 260 µm. The filtrate was washed in 0.9% NaCl solution and once more passed through a steel sieve (pore diameter 100 µm) in order to separate crude material. The filtrate was passed through a nylon sieve (pore diameter 70 µm). This step separated the glomeruli, which were retained in the sieve, from the smaller tubuli. The suspension of glomeruli found by light microscopy contained about 95% of the glomeruli. They were suspended in 0.32 M sucrose solution (15 ml/g glomeruli) and homogenized at 4°C in a Potter-Elvejhem homogenizer. The basement membranes were isolated by discontinuous sucrose gradient ultracentrifugation. The solutions used, besides the homogenization medium, were 1.1 M, 1.3 M, 1.5 M, 1.65 M, 1.775 M sucrose. The gradient was centrifuged for 45 min at 75,000 g. The pellet contained the basement membrane fraction, which was incubated twice with DNAse for 10 min at 37°. The purity of the basement membrane fraction was determined by light microscopy and by determination of the concentration of the amino acid, hydroxyproline (Stegemann, 1958).

Immunization of rabbits and production of a polyclonal basement membrane antiserum

Rabbits were immunized according to the following schedule: firstly, 500 µg of basement membranes in complete Freund's adjuvant, subcutaneously; secondly, 500 µg of basement membranes in incomplete Freund's adjuvant, intramuscularly 4 weeks later, and finally, another 4 weeks later, 500 µg of basement membrane suspension, intraveneously. The animals were bled 5 and 9 weeks after the first injection.

Immunization of Balb/c mice, cell fusion and culture conditions

Six-week-old female Balb/c mice (n = 6) were immunized 3 times with 250 µg basement membrane suspension. The first injection was intraperitoneal with complete Freund's adjuvant, the second injection 4 weeks later with incomplete Freund's adjuvant, and the third injection were intravenous without adjuvant 4 days before cell fusion. Spleen cells were prepared and fused with the non-secreting mouse myeloma cell line, SP2/0-AG14 (Schulman et al., 1978) using polyethyleneglycol 6000 (Merck/Schuchardt, München, F.R.G.) as described (Köhler and Milstein, 1975). These cells and the hybridomas produced from them were cultivated in RPMI 1640 containing 40 mg/l folic acid, 15% calf serum, 2mM glutamine, 12.5 µg/ml streptomycin and 12.5 IU/ml penicillin G.

Two cell fusions were carried out: The first cell fusion of 2.4×10^9 spleen cells from 3 Balb/c mice with 2.37×10^8 SP2/0-AG14 myeloma cells; the second fusion of 1.4×10^9 spleen cells form 3 Balb/c mice with 2.37×10^8 SP2/0-AG14 myeloma cells. The fused cells were plated out on 2 x 12 96-well micro-well plates (Costar, Cambridge, Mass., USA). The plating medium was the same as the above-mentioned, except for the addition of azaserin (10^{-5} M) and hypoxanthine (5×10^{-5} M).

Cloning of positive hybrids was carried out in 96-well-plates dispersing 150 µl of cell suspension containing 3 cells/ml into each well. The same medium (without azaserin) was used.

Indirect immunofluorescence

Rat kidneys, 18-day-old rat embryos, and bovine and human tissue samples were snap-frozen in liquid nitrogen and cut at -20°C using a SLEE cryostat. Sections (10 µm) were air-dried on glass slides and incubated for 30 min at room temperature, either with monoclonal supernatants or with rabbit antiserum at appropriate dilutions. They were washed three times with PBS and then reacted with fluorescein-isocyanate (FITC)-labelled rabbit anti-mouse Ig or goat anti-rabbit Ig, respectively (Paesel GmbH, Frankfurt, F.R.G.) in order to detect bound antibody. The slides were once more washed three times with PBS, cover slips were mounted and the sections were inspected under a Zeiss microscope equipped with epifluorescent optics.

Control method

Normal rabbit serum and supernatant of SP2/0-AG14 cell line were used instead of rabbit anti-basement membrane antiserum or supernatants or 'ascites of monoclonal antibodies, respectively.

ELISA

The ELISA used was the procedure of Engwall and Perlmann (1972) as modified by Gosslau and Barrach (1979). Microtiter plates (Titertek Dynatech, Denkendorf, F.R.G.) were coated with collagen type IV, heparan sulphate proteoglycan and laminin, all of which were gifts from Dr. J. Barrach, Institut für Toxikologie und Embryonalpharmakologie, Freie Universität Berlin, and fibronectin (Boehringer Mannheim GmbH., Mannheim, F.R.G.). Each well was coated with 5 µg of one of the above-mentioned substances in 100 µl coating buffer and incubated for 6 days at 4°C. The coated plates were washed three times

with PBS and incubated with 100 µl of culture supernatant, ascites (dilutions from 1:30 to 1:150) and rabbit antiserum against collagen type IV, heparan sulphate proteoglycan and laminin (dilution each 1:400) for 45 min at room temperature on a microshaker. The antisera were a gift from Dr. J. Barrach. After washing three times, the wells were covered with 100 µl of peroxidase-conjugated rabbit anti-mouse IgM antibody (dilution 1:100 in PBS/BSA) or peroxidase-conjugated goat anti-rabbit polyvalent antibody (Miles Laboratories Ltd., Wien, Austria) (dilution 1:500 in PBS/BSA), respectively. After incubation at room temperature for 45 min on a micro-shaker, the microplates were again washed as described above and incubated with 100 µl 5-amino 2-hydroxybenzoic acid reagent. After 45 min, the reaction was stopped with 1 N NaOH.

Results

Rabbit anti-glomerular basement membrane antiserum

The polyclonal antiserum against rat glomerular basement membrane was analyzed immunohistochemically on frozen sections of rat kidneys. The staining patterns corresponded to those of basement membranes of rat glomeruli, Bowman's capsules, tubular basement membranes and interstitial structures. The antiserum also showed a mesangial pattern in glomeruli (Fig. 1A).

Production and characterization of monoclonal antibodies

Two cell fusions were carried out with spleen cells from 6 female Balb/c mice immunized with purified rat glomerular basement membranes with cells from the mouse myeloma SP2/0AG-14 cell line. Hybridoma growth was found in 129 of the 2,304 microwells. The supernatants of these hybrid colonies were analyzed biochemically in frozen sections of rat kidneys. Production of antibodies against renal antigens was observed in 11 hybrid colonies. Five out of these 11 hybrid colonies (Z 1, Z 2, Z 3, Z 4, Z 5) produced antibodies against cell nuclei of cells throughout the kidney (Fig. 1B). All 5 hybrids showed identical staining patterns. One hybrid colony (T 1) developed antibodies against vesicle-like structures within the tubular cells (Fig. 1C).

The staining patterns of these 6 hybrid colonies differed from those of the rabbit antiserum, which did not react with cell nuclei or vesicular structures in tubular cells. Five hybrid colonies produced antibodies against basement membranes of rat kidneys (BM O II, O IV, O VII, M II, M III). A large number of clones were obtained from the 5 anti-basement membrane antibody-producing hybridomas. The monoclonal antibodies BM O II, BM O IV, BM O VII, BM II and BM M III were stable and the antibodies were analyzed. For Ig subclass determination of the antibodies secreted by BM O II, BM O IV, B M O VII, BM M II and BM M III hybridoma clones, the cells were grown in the presence of [^{35}S] methionine for several hours. The secreted proteins were analyzed by the Ouchterlony (1962) double-diffusion procedure against subclass specific anti-mouse Ig antibodies from rabbit and by SDS-PAGE. The results are represented in Fig. 2. All 5 hybridomas produced antibodies of the IgM subclass.

Immunohistochemical characterization of monoclonal antibodies in frozen sections of rat kidneys

The staining pattern of the 5 monoclonal antibodies are shown in Table 1. BM O II showed a stringent specificity for glomerular basement membrane, which was stained in a linear pattern (Fig. 3A). Monoclonal antibody BM O VII reacted with tubular basement membranes, the Bowman's capsule and with fibrillar structures in rat kidney (Fig. 3B). Blood vessels, mesangium and glomerular basement membrane were not marked. BM O IV, BM M II and BM M III showed an identical immunohistochemical phenotype on rat kidneys. They reacted with glomerular basement membrane, tubular basement membrane and blood vessels, as well as with mesangial cells. Glomerular and tubular basement membranes showed a diffuse, partly discontinuous linear staining pattern (Fig. 3C).

Other tissues

Mouse antibody BM O II showed a stringent species specificity. No antibody binding was found in other tissues. Different results were obtained with BM O VII, BM O IV, BM M II and BM M III. They had a wide range of reactivity with basement membranes in different tissues and were not limited to one species (Table 2). On frozen sections of rat embryos, BM O VII reacted with tissue separating organs from their surrounding, e.g. the capsule of the liver, peritoneum, perichondrium or dura (Fig. 4A). The connective tissue associated with the intervertebral disk (Fig. 4A), the heart valves, the endocardium and the tunica muscularis of the intestine were also marked by BM O VII, BM M III reacted with cartilage cells (Fig. 4b) as well as with the tunica muscularis and tunica mucosa (Fig. 4C) of the intestine from rat embryos. The staining patterns on frozen sections of human and bovine kidney were identical with those of rat kidney (data not shown). BM M II and BM O IV staining did not differ from that of BM M II. They also showed the same reaction to human and bovine kidney (data not shown).

Monoclonal supernatants and ascites in appropriate dilutions were assayed on ELISA with specifically purified components of the basement membrane such as collagen type IV, laminin, heparan sulphate proteoglycan and fibronectin. None of the described antibodies reacted with these known basement membrane components. Specific antisera from rabbits were used as positive control.

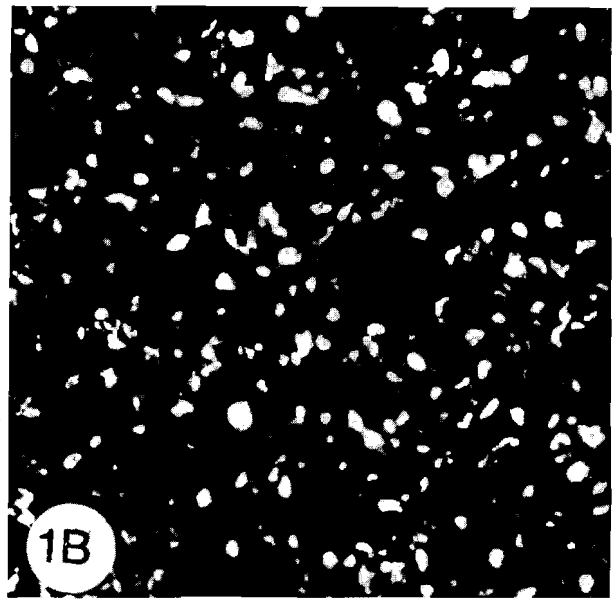
Table 1. Reactivity of monoclonal antibodies with rat kidney sections.

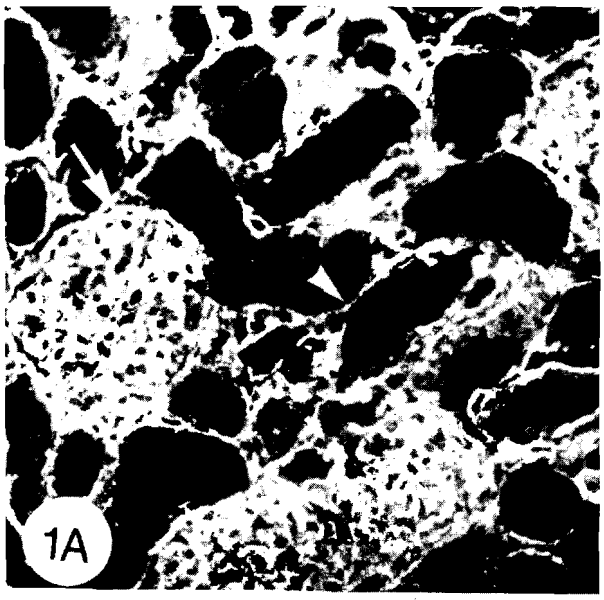
	Sites reactive by indirect immunofluorescence				
	GBM	TBM	Blood vessels	Bowman's capsule	Mesangium cells
BM O II	+	–	–	–	–
BM O VII	–	+	–	+	–
BM O IV	+	+	+	+	+
BM M II	+	+	+	+	+
BM M III	+	+	+	+	+

Abbreviations: GBM – glomerular basement membrane; TBM – tubular basement membrane

Table 2. Reactivity of monoclonal antibodies with rat embryonic sections.

	Sites reactive by indirect immunofluorescence		
	Intestine	Dura mater, Intervertebral disc	Cartilage cells
BM O II	–	–	–
BM O VII	+	+	+
BM O IV	+	–	+
BM M II	+	–	+
BM M III	+	–	+





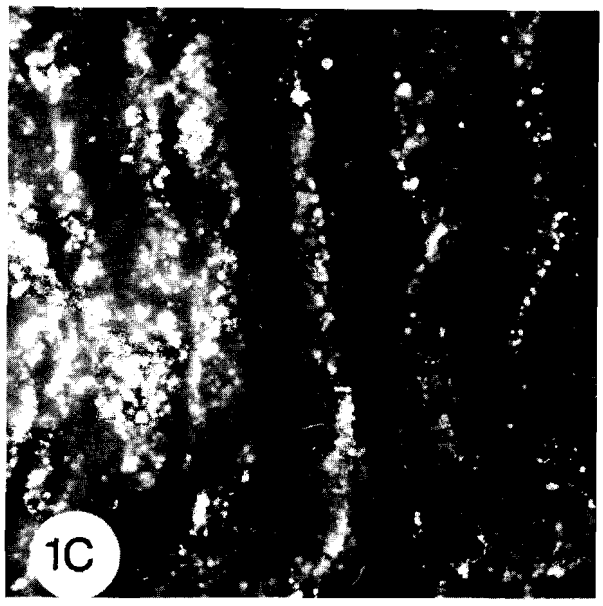


Fig. 1. Indirect immunofluorescence of rat kidney sections stained with A: Rabbit antiglomerular basement membrane antiserum. The antiserum reacts strongly with the glomerular (arrow) and tubular (arrow head) basement membrane. Also note the reactivity of Bowman's capsule, mesangium and the interstitial structure. × 350 B: Hybridoma supernatant from Z1. The antibodies react with cell nuclei throughout the kidney × 350. C: Hybridoma supernatant from T1. The antibodies react with vesicle-like structures within the tubular cells. × 450

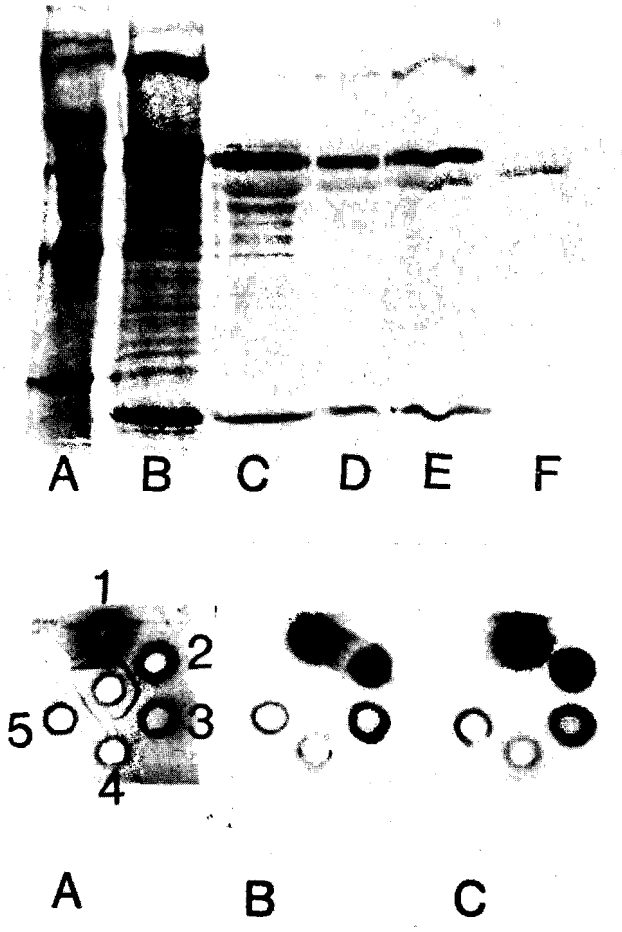
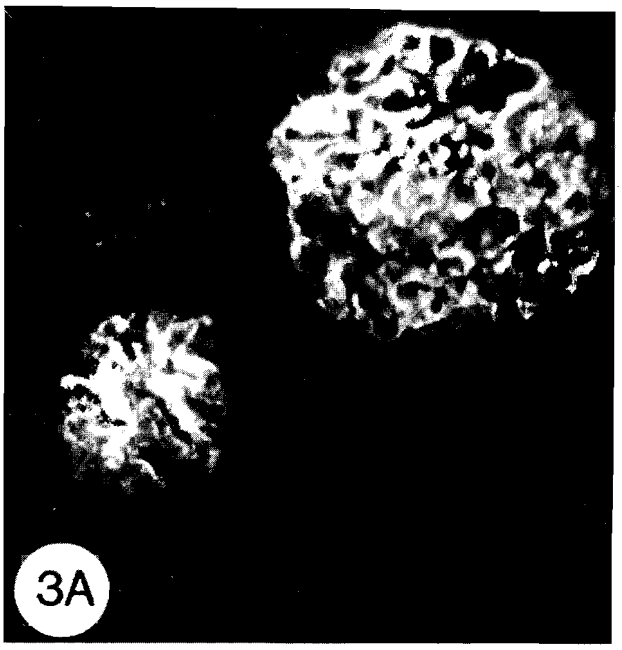
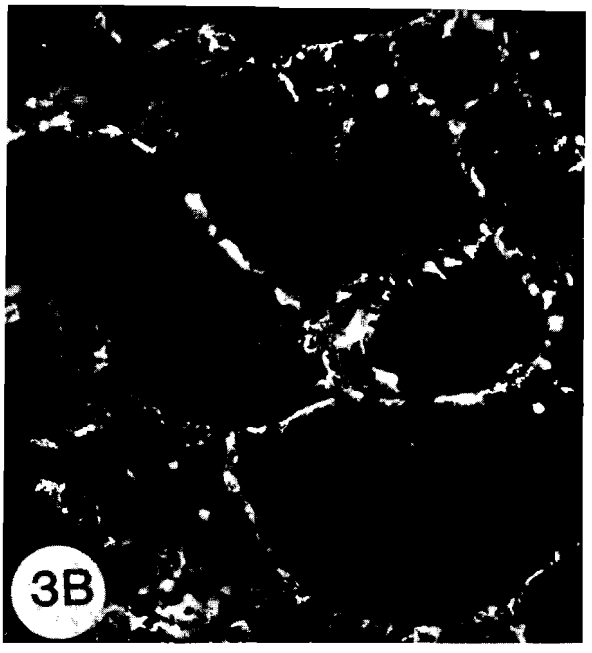


Fig. 2. Analysis of immunoglobulin subclass. (Upper). Autoradiography of secreted ^{35}S-labelled proteins from five monoclonal antibody-producing cell lines. Analysis in 10% (wt/vol) acrylamide slab gels in the presence of $NaDoSO_4$. A, M_r stardards: carbonic anhydrase (30,000), ovalbumin (46,000), bovine serum albumin (69,000), phosphorylase b (92,500) and myosin (200,000); B, BM O II; C, BM O VII; D, BM O IV; E, BM M II; F, BM M III. The M_r of the heavy chains of all monoclonal antibodies were in the 70,000 range. (Lower). Autoradiography of Ouchterlony double-diffusion analysis of the supernatants of ^{35}S-labelled BM O II (1), BM O VII (2), BM O IV (3), BM M II (4) and BM M III (5). Precipitation lines were observed with anti-mouse-IgM-antiserum (A) with all five ^{35}S-labelled monoclonal antibodies. No precipitation lines were observed with anti-mouse-IgG1-, anti-mouse-IgG2a-, anti-mouse-IgG2b-, anti-mouse-IgG3-, anti-mouse-IgE- and anti-mouse-IgA-antiserum. Antisera in the central wells were: A, anti-mouse-IgM-antiserum; B, anti-mouse-IgA-antiserum; C, anti-mouse-IgG2a-antiserum. The data of the other anti-Ig-antisera are not shown.





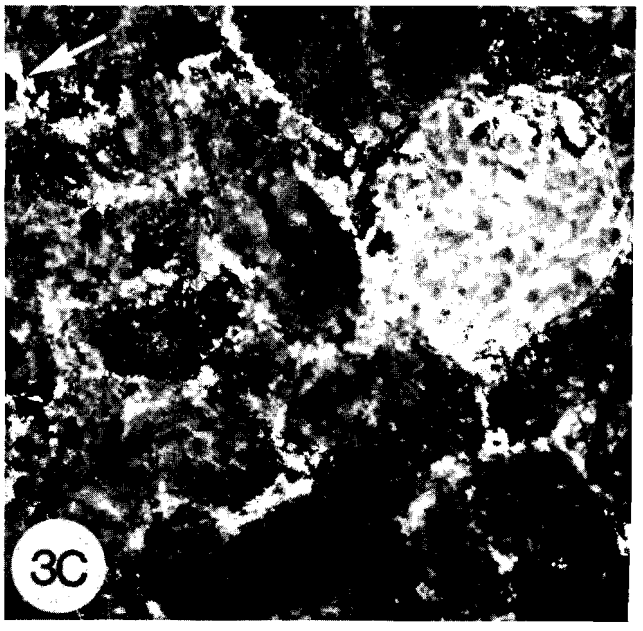
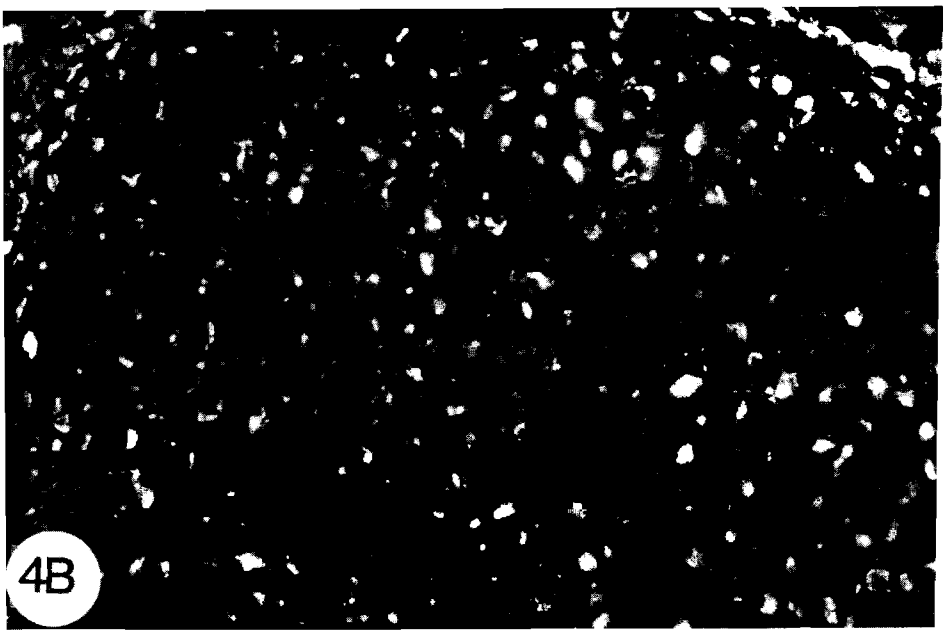
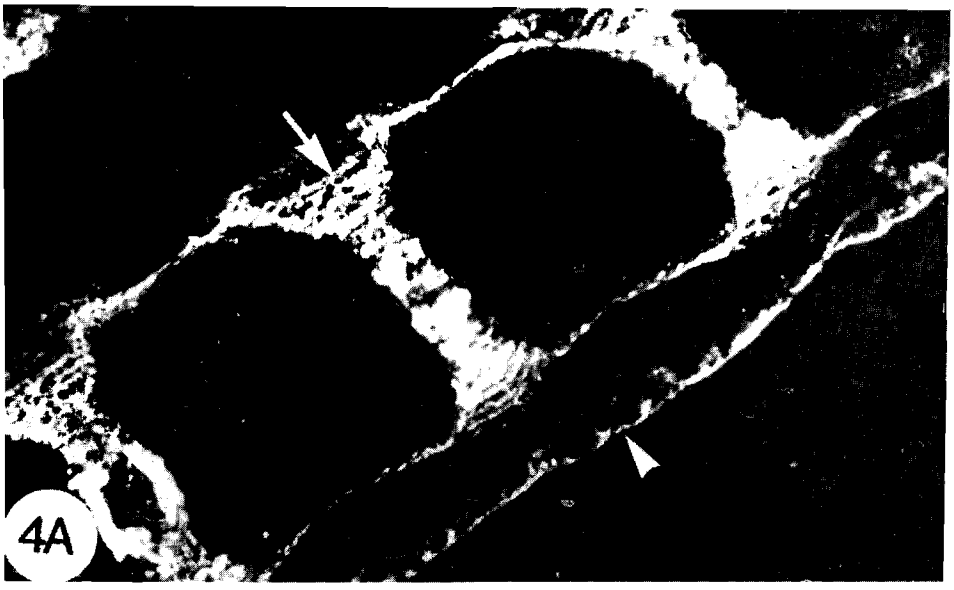


Fig. 3. Indirect immunofluorescence of rat kidney sections stained with monoclonal antibodies. A: BM O II shows exclusively a linear staining pattern in glomerular basement membrane × 350. B: BM O VII reacts strongly with tubular basement membrane and fibrillar structures × 450. C: BM O III stains tubular and glomerular basement membranes as well as mesangium cells and blood vessels (arrow). × 350





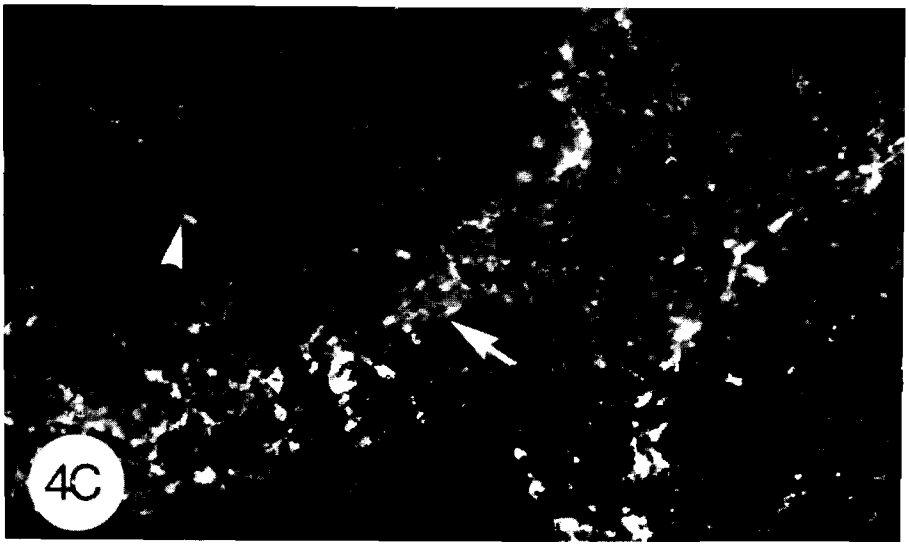


Fig. 4. Indirect immunofluorescence of rat embryo sections stained with monoclonal antibodies. A: BM O VII on embryonic vetebral column. BM O VII reacts with the intervertebral disc (arrow) and dura (arrow head). × 140. B: BM III on cartilage. BM M III shows a staining pattern with all cartilage cells. × 350. C: BM M III on embryonic intestine. BM M III reacts with the tunica muscularis (arrow) as well as with the tunica mucosa (arrow head). × 240

Discussion

Glomerular basement membranes of kidneys from adult rats, which were used for immunization, were purified to a high degree and isolated by different steps of sieving and sucrose gradient ultracentrifugation. Without any further biochemical treatment of the basement membranes we injected the basement membrane suspension into rabbits and mice in order to develop poly- and monoclonal antibodies. In other studies, basement membranes were treated with detergents, collagenase, sonification or citrate extraction and used as antigens. By these treatments, basement membrane components may be either lost or destroyed. In this study, production and characterization of monoclonal antibodies against glomerular basement membranes of rats was initiated to discover so far unidentified components of basement membranes, even possibly not yet discovered. The morphological characterization of the antibody specificity was determined by the reactivity of monoclonal supernatants on frozen sections of kidneys from adult rats. Six out of eleven monoclonal antibodies did not react with basement membrane antigens, but with cell nuclei (Z 1 - 4) or with vesicle-like structures in tubular cells. These results indicated that the basement membranes were obviously not purified to homogeneity. On the other hand, it is of interest that, in polyclonal antiserum of rabbits, there was no reaction with either cell nuclei or with antigens within the tubular cells, although the same basement membrane preparation was used for immunization.

Five out of eleven monoclonal antibodies reacted with basement membranes of rat kidney. In comparison with the polyclonal rabbit anti-glomerular basement membrane antibodies, monoclonal antibodies did not show a very intense staining of basement membranes. This phenomenon has been observed before (Wan et al., 1984) and might be due to the fact that the polyclonal antiserum possesses more different antibodies with varying specificity. Supernatants of monoclonal antibodies were also investigated by ELISA, where known components of basement membranes were assayed as possible antigens. None of the five monoclonal antibodies reacted with laminin, collagen type IV, heparan sulphate proteoglycan or fibronectin. In further investigations other purified basement membrane components such as nidogen, entactin or sialoproteins should be assayed in order to verify that the antibodies recognize so far unidentified components of the basament membrane.

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