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Nephritogenicity and α-chain composition of NC1 fractions of type IV collagen from bovine renal basement membrane

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Abstract Nephritogenicity (anti-GBM-nephritis-inducing activity) and α-chain composition of globular-domain (NC1) fractions of type IV collagen from bovine renal, pulmonary, and placental basement membranes (BMs) was examined by injecting these fractions with adjuvant into WKY/NCrj rats and by Western blotting using epitope-defined monoclonal antibodies to the six different α chains of type IV collagen. A purified nephritogenic fraction from renal BM contained α1-α6(IV)NC1, whereas a non-nephritogenic fraction contained only α1-α2(IV)NC1. Renal and pulmonary NC1 had strong nephritogenic activity; placental NC1 had weak activity. The renal and pulmonary fractions contained α1-α6(IV)NC1, and the placental fraction had a large amount of α1-α2(IV)NC1 and a very small amount of α3-α6(IV)NC1. Immunohistochemical study of bovine renal BM with the monoclonal antibodies revealed that bovine glomerular BM contained α1-α5(IV) chains, but not the α6(IV) chain. The absence of α6(IV) chain in glomerular BM in bovine and in humans indicates that α6(IV) chain is not a target antigen of anti-GBM nephritis. Nephritogenicity is apparently a property of α3-α5(IV)NC1.

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Introduction

Anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM nephritis) is an autoimmune disease induced by autoantibodies to glomerular basement membrane (GBM) [36]. The active type of experimental anti-GBM nephritis was first reported in 1962 by Steblay [30], who injected with multiple doses of insoluble GBM emulsified with adjuvant to sheep. Improved active-type models induced in rats by a single injection of an emulsion of soluble nephritogenic antigen from GBM and adjuvant have now become popular [19, 21–23, 25]. Passive types of experimental anti-GBM nephritis induced by the injection of homologous polyclonal [24] or monoclonal [26, 31] antibodies to GBM or type IV collagen have also been reported.

Recent studies on basement membrane have revealed that its main component is type IV collagen and that there are six different isoforms, α1(IV) to α6(IV) chains of this type of collagen. The complete amino acid sequences of the human 6 chains have already been deduced [1, 2, 6, 14, 15, 20, 29, 37, 39]. A globular domain (NC1 domain) at the carboxyl terminus of the type IV collagen α3 chain, α3(IV)NC1, is thought to be an antigen responsible for anti-GBM nephritis [3, 4, 8–10, 17, 28, 33–35]. This is based on the fact that autoantibodies to α3(IV)NC1 can be detected in sera of patients with anti-GBM nephritis by Western blotting and by enzyme immunoassay using recombinant α3(IV)NC1 protein. In addition to α3(IV)NC1, α4(IV)NC1 is also thought to be an antigen responsible for anti-GBM nephritis, since experimental anti-GBM nephritis can be induced in rats by injection with a synthetic peptide having part of the sequence of the human α3(IV)NC1 or α4(IV)NC1 chain [31].

We have recently developed a novel method for making rat monoclonal antibodies by using lymph-node lym-

phocytes as a source of B cells [11]. The lymphocytes are taken from enlarged medial iliac lymph nodes of rats following injection of emulsified antigen into their hind footpads. The frequency of positive hybridomas producing antibodies to the immunogen is about 10 times that obtained by the conventional method using mouse or rat splenic lymphocytes as a source of B cells. By this method we have established 13 α-chain-specific epitope-defined monoclonal antibodies to the NC1 domain of type IV collagen α-chains by using synthetic peptides as immunogens [18, 27]. Nine of them can be used for identification of bovine type IV collagen α-chains.

This paper describes the nephritogenicity of NC1 fractions from bovine kidneys, lungs, and placentas, and characterization of the NC1 fractions by use of the monoclonal antibodies. The result demonstrates that nephritogenicity of a fraction is related to the amount of α3-α5(IV)NC1 contained in it.

Materials and methods

Female WKY/NCrj inbred rats purchased from Charles River Japan (Yokohama, Japan) were used. The rats were housed in polycarbonate plastic cages containing wood shavings as bedding. The animal experiments in the present study were conducted according to the Guidelines for Laboratory Animal Experiments of Shigei Medical Research Institute.

A nephritogenic fraction (P2-P3 fraction) and a non-nephritogenic fraction (P2 fraction) were purified by using antibody-coupled affinity columns such as have been described previously [25].

Bovine renal basement membrane (RBM), pulmonary basement membrane (LBM), and placental basement membrane (PBM) were prepared by the method described previously [25]. Lyophilized basement membrane was solubilized with collagenase (Seikagaku, Tokyo, Japan) at 47°C for 20 h by a method similar to that of Butkowski et al. [3]. After solubilization the sample was centrifuged at 27,000 *g* for 10 min and insoluble material was removed. The supernatant material was dialysed against distilled water for 8 h, lyophilized, dissolved in phosphate-buffered saline (PBS), and dialysed against PBS overnight. The dialysed material was centrifuged to remove insoluble material formed during the dialysis. The supernatant was then applied to a gel filtration column of Ultrogel AcA 34 (26×950 mm) at a flow rate of 30 ml/h. The main peak fraction (tube numbers 37–42, 10 ml in each tube), whose peak tube number was the same as that of bovine serum albumin, was collected and concentrated. These NC1 fractions obtained from RBM, LBM, and PBM were named RBM-U, LBM-U and PBM-U, respectively.

The nephritogenicity of RBM-U, LBM-U, and PBM-U was examined by injecting the fractions into rats. Each fraction was emulsified with an equal volume of Freund's complete adjuvant and injected into the hind footpads of 8-week-old female

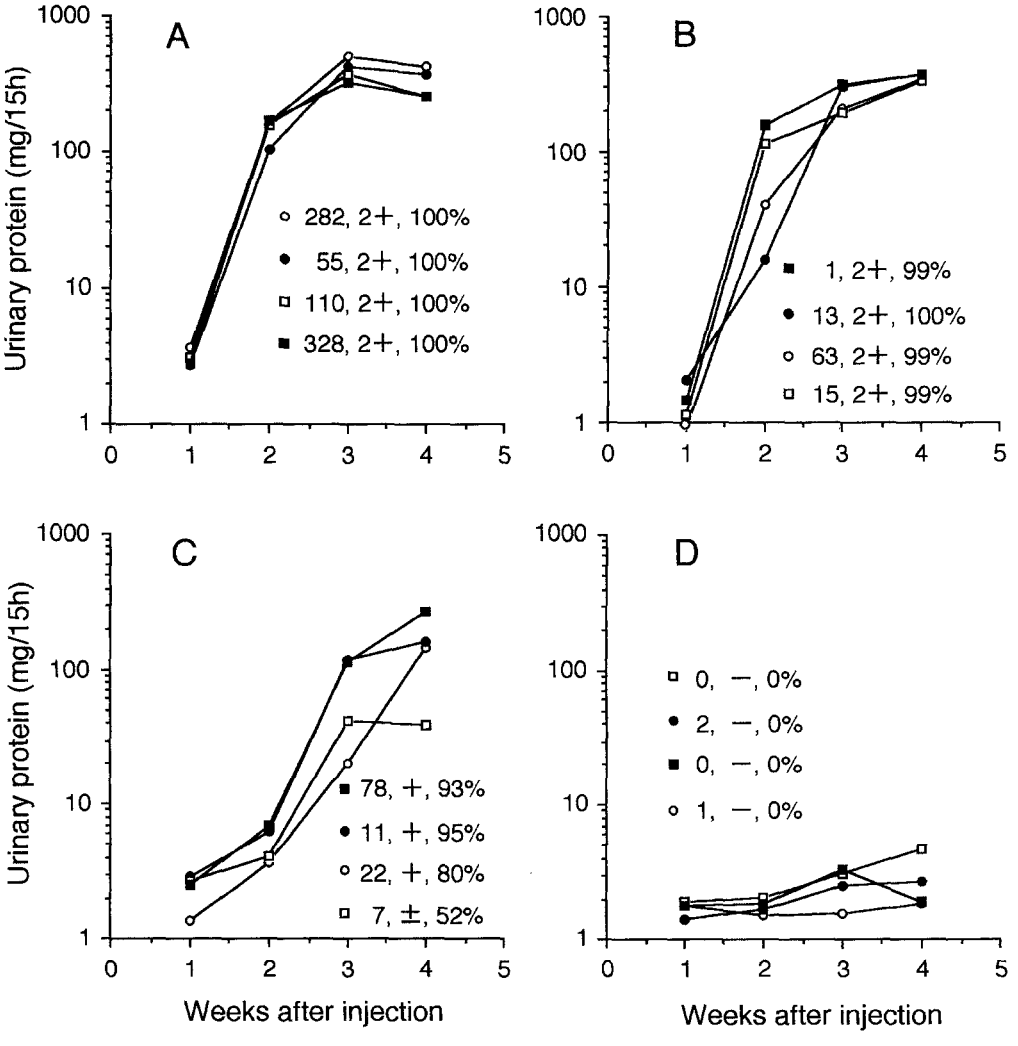

Fig. 1 Time-course of proteinuria of rats following injections of 300 μg of collagenase-solubilized NC1 fraction from bovine renal (**A**) pulmonary (**B**) and placental (**C**) basement membrane or phosphate-buffered saline only (**D** control). Number of petechiae on the surface of the lung, intensity of direct immunofluorescence of IgG on the glomerular basement membrane (GBM), and percentage of abnormal glomeruli in the kidney 4 weeks after the injection are expressed

WKY/NCrj rats. Urine (from 6:00 p.m. to 9:00 a.m.) was collected weekly for 4 weeks after injection and assayed for urinary protein by a method employing 3% sulfosalicylic acid [22] and for haematuria by use of test papers (Hemastix, Miles-Sankyo, Tokyo, Japan). The rats were sacrificed 4 weeks after the injection. Samples of renal tissue were fixed in Dubosq-Brazil fixative, embedded in paraffin, sectioned at 3 μm, and stained with PAS and PAM. For direct immunofluorescence, 4-μm cryostat sections of the kidney were stained with FITC-conjugated antibody to rat IgG and C3 (Cappel Lab., USA).

SDS-PAGE was performed according to the method of Laemmli [13] with SDS-containing 5% stacking and 11.5% homogeneous running polyacrylamide slab gels. Samples were applied to the gels without reduction. The gels were stained with Coomassie Brilliant Blue. Standards (Pharmacia, Uppsala, Sweden) used to calculate molecular mass included phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

Western blotting was carried out by transferring the electrophoresed materials from SDS-PAGE gels onto 0.22-μm pore polyvinylidene difuloride (PVDF) membrane (Nihon Millipore, Yonezawa, Japan). After the transfer, the membrane was blocked by incubation with 3% bovine serum albumin (Sigma, St. Louis, Mo.) in 50 mM Tris-HCl buffer containing 150 mM NaCl (TBS), pH 7.5, for a minimum of 8 h. The membrane was cut into 5-mm-wide narrow strips and incubated with culture supernatant of monoclonal antibody for 2 h. The strips were rinsed with TBS containing 0.05% Tween-20 (Tw-TBS) and incubated for 1 h at room temperature with peroxidase-conjugated anti-rat IgG (Dako A/S, Glostrup, Denmark) diluted with TBS containing 3% bovine serum albumin, which procedure was followed by extensive washing with Tw-TBS. The blots were developed with H_2O_2-diaminobenzidine as substrate.

Enzyme-linked immunosorbent assay (ELISA) was performed as described previously [26].

Monoclonal antibodies to human α1-α6(IV)NC1 used were the epitope-defined ones established in our laboratory [27] by the rat lymph node method [11]. Monoclonal antibodies used were as follows: H11 for α1(IV)NC1, H21 for α2(IV)NC1, H31 for α3(IV)NC1, H43 for α4(IV)NC1, H52 for α5(IV)NC1, and H61 for α6(IV)NC1. These antibodies stained human cryostat sections very well, although the staining ability of H61 in Western blotting was not strong [27]. Another monoclonal antibody, H65, which was against human α6(IV) COL domain, was used for staining of bovine cryostat sections [18].

Fresh bovine kidney tissues in OCT compound (Miles Inc., Elkhart, Indiana, USA) were snap-frozen in liquid nitrogen. They were cut into 4-μm sections and stained with the monoclonal antibodies by the indirect immunofluorescence method [27].

Results

Nephritogenicity of P2-P3 and P2 fractions from RBM

Four rats that each received 30 μg of the P2-P3 fraction of renal basement membrane had proteinuria with haematuria starting 2 weeks after the injection, whereas 4 rats that received 100 μg of the P2 fraction had no proteinuria following the injection. Linear immunofluorescence along the GBM was observed in the rats injected with the P2-P3 fraction, but not in the rats that received the P2 fraction. Very severe histological changes were seen in the rats to which the P2-P3 fraction was injected, whereas no histological change was seen in the animals that received the P2 fraction.

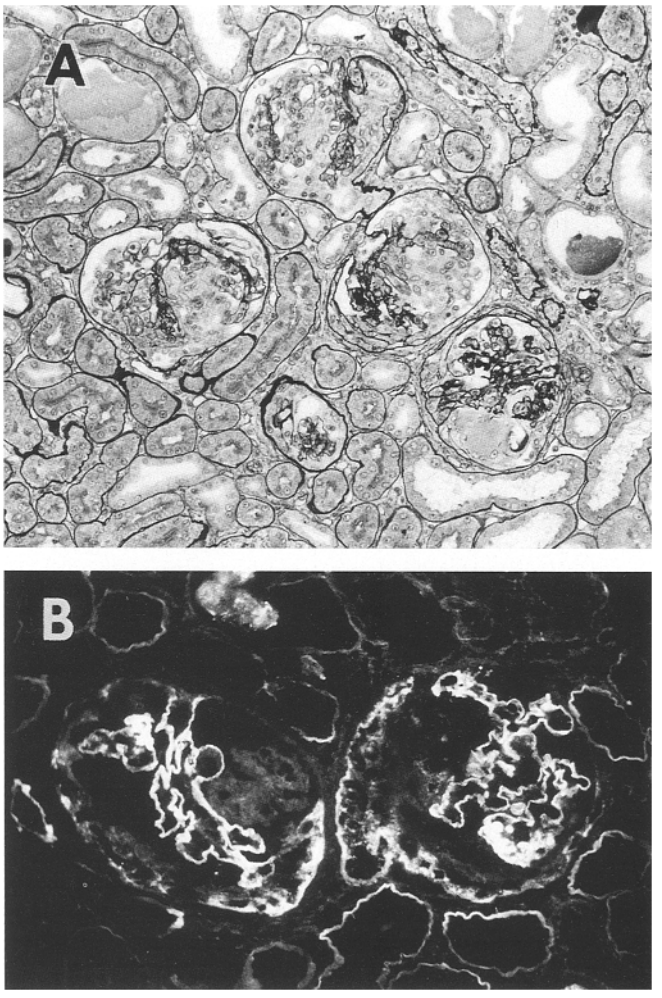

Fig. 2A, B Photomicrographs of kidney from rat injected with 300 μg of collagenase-solubilized bovine nephritogenic antigen (RBM-U) 4 weeks after injection. **A** PAM staining. Severe damage is seen in all glomeruli.×135. **B** Direct immunofluorescence with FITC-conjugated anti-rat IgG. Linear IgG deposition is observed along the GBM. ×200

Nephritogenicity of the NC1 fractions from kidneys, lungs, and placentas

Yields of the NC1 fractions from 5 g of lyophilized basement membrane from kidneys, lungs, and placentas were 141 mg, 46 mg, and 40 mg, respectively. Rats received injections of 300 μg of RBM-U, LBM-U, or PBM-U. The time course of urinary protein, number of petechiae on the surface of the lung at sacrifice, intensity of immunofluorescence on the GBM, and percentage of damaged glomeruli 4 weeks after the injection are shown in Fig. 1.

In rats that received injections of RBM-U and LBM-U, proteinuria with haematuria started 2 weeks after the injection, whereas in those to which PBM-U was injected, the proteinuria started 3 weeks after the injection. Direct immunofluorescence on the GBM was more intense following RBM-U or LBM-U than after PBM-U. Histological changes seen in rats that received injections of

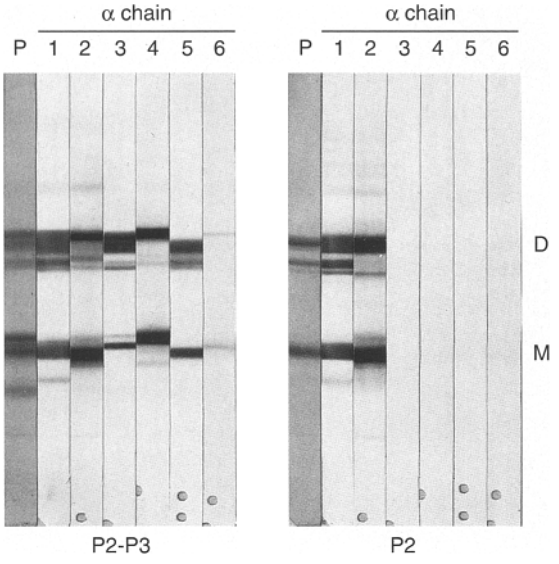

Fig. 3 Western blotting pattern of nephritogenic (*P2-P3*) and non-nephritogenic (*P2*) fractions from bovine renal basement membrane. The monoclonal antibodies used: H11 for α1(IV), H21 for α2(IV), H31 for α3(IV), H43 for α4(IV), H52 for α5(IV), and H61 for α6(IV). (*D* Dimer, *M* monomer, *P* protein staining)

RBM-U or LBM-U were more severe than those in the rats injected with PBM-U. Mild to moderate pulmonary haemorrhage was observed in the rats, and it was more intense in those that had received RBM-U. This result indicated that nephritogenic antigen was present in RBM, LBM, and PBM but that the amount in PBM was presumably very small.

Histological findings in glomerulonephritis induced in the present study

The kidneys of the nephritic rats 4 weeks after injection were pale and swollen. The weight of the kidneys affected by severe disease was about twice that of normal kidneys. Light microscopy showed severe glomerular changes, such as endocapillary hypercellularity of mononuclear cells, capsular adhesion, cellular crescent formation, collapse of capillary tufts, and protein droplets in epithelial cells of rats treated with RBM-U (Fig. 2A). No glomerular infiltration of polymorphonuclear leucocytes was found. In severe cases some tubules were dilated and some were atrophied, and hyaline casts within the lumina of tubules were prominent. Use of the direct immunofluorescence method revealed the intense linear deposits of rat IgG along the GBM of animals that had received injections of RBM-U (Fig. 2B). Pulmonary haemorrhage was also found in rats that had received RBM-U.

Analyses of P2-P3 and P2 fractions by Western blotting with α-chain-specific monoclonal antibodies

Western blotting patterns of P2-P3 and P2 fractions are shown in Fig. 3. The nephritogenic P2-P3 fraction contained α1-α6(IV)NC1, whereas the non-nephritogenic P2 fraction contained only α1 and α2(IV)NC1. It is thus clear that α3-α6(IV)NC1 are candidates for the functions of nephritogenic epitopes.

Analyses of RBM-U, LBM-U, and PBM-U by Western blotting with α-chain-specific monoclonal antibodies

Western blotting patterns of RBM-U, LBM-U, and PBM-U are shown in Fig. 4. Staining with the monoclonal antibodies to NC1 domains showed that the nephritogenic RBM-U and LBM-U contained α1-α6(IV)NC1, whereas the weakly-nephritogenic PBM-U contained mainly α1(IV)NC1 and α2(IV)NC1. The molecular masses of monomers of these bands were as follows: α1(IV)NC1, α5(IV)NC1, and α6(IV)NC1, 26 kDa; α2

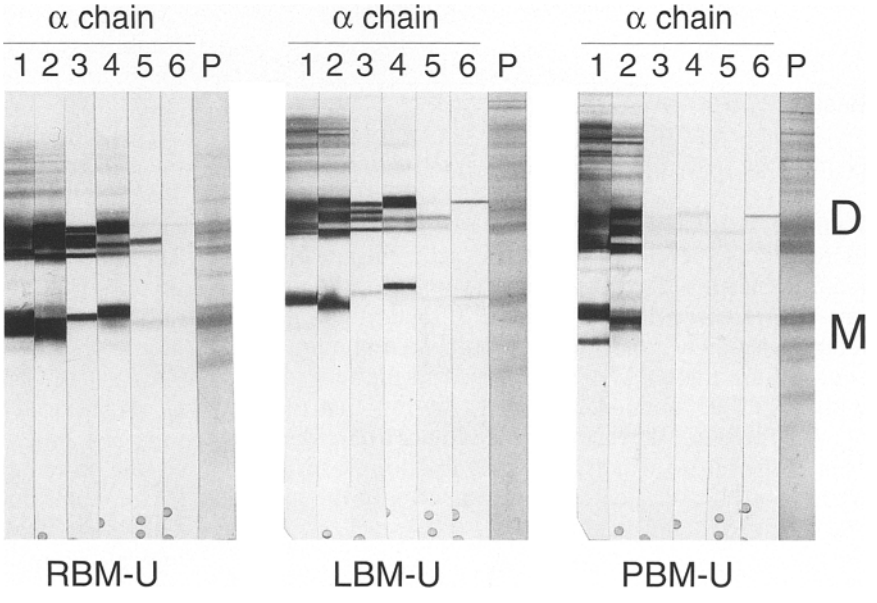

Fig. 4 Western blotting pattern of NC1 fractions from bovine renal (*RBM-U*), pulmonary (*LBM-U*), and placental (*PBM-U*) basement membranes. The monoclonal antibodies used: H11 for α1(IV), H21 for α2(IV), H31 for α3(IV), H43 for α4(IV), H52 for α5(IV), and H61 for α6(IV)

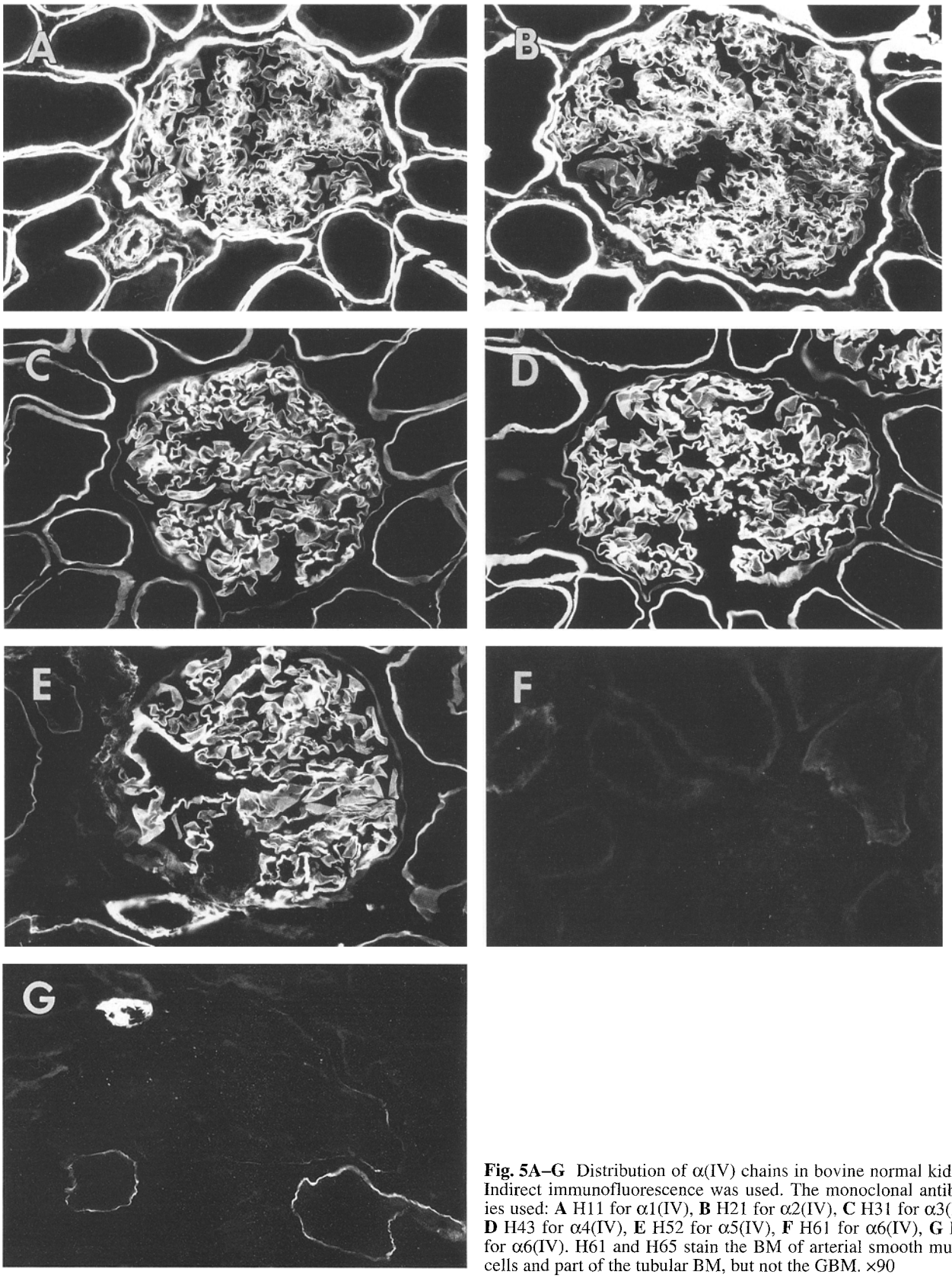

Fig. 5A–G Distribution of α(IV) chains in bovine normal kidney. Indirect immunofluorescence was used. The monoclonal antibodies used: **A** H11 for α1(IV), **B** H21 for α2(IV), **C** H31 for α3(IV), **D** H43 for α4(IV), **E** H52 for α5(IV), **F** H61 for α6(IV), **G** H65 for α6(IV). H61 and H65 stain the BM of arterial smooth muscle cells and part of the tubular BM, but not the GBM. ×90

(IV)NC1, 25 kDa; α3(IV)NC1, 27 kDa; and α4(IV)NC1, 28 kDa. Monoclonal antibodies stained not only the monomers but also peptide dimers of about 50 kDa.

Distribution of α(IV) chains in bovine kidneys

Bovine kidney sections stained with the monoclonal antibodies to α1-α6(IV) are shown in Fig. 5. H11 for the α1(IV) chain and H21 for the α2(IV) chain stained the bovine kidney with the same pattern. They stained the subendothelial region of the GBM contiguous with the mesangial matrix and the mesangial matrix. They also stained the Bowman's capsule BM, the tubular BM, and the vascular BM. H31 for α3(IV) and H43 for α4(IV) stained bovine kidney with the same pattern but one that was different from that for α1(IV) and α2(IV). They stained the GBM, the tubular BM, and the Bowman's BM in a linear pattern. However, they did not stain the mesangial matrix or the vascular BM. H52 for α5(IV) stained bovine kidney in a similar pattern to that of α3(IV) and α4(IV), but it also stained the BM of arterial smooth muscle cells. H61 for α6(IV) stained only the BM of arterial smooth muscle cells and, faintly, part of the tubular BM. H65, which is against the third imperfection of the triple helical domain of the α6(IV) chain, stained in the same manner as H61.

Discussion

A fraction containing a nephritogenic antigen that induces anti-GBM nephritis in rats has been purified from bovine renal basement membranes [25]. Although it has been demonstrated that the fraction contains the NC1 domains of the α-chains of type IV collagen, its α chain composition has not yet been completely analysed. This is because the amino acid sequences of newly found chains of type IV collagen such as α5(IV) and α6(IV) chains have only recently been deduced [7, 32, 38] and because no reliable monoclonal antibodies to them have been available. The monoclonal antibodies used in this study were epitope-defined antibodies [27] established by a newly developed method designated the rat lymph node method [11]. Although they were raised against human immunogens, they also react with bovine antigens because α-chains of bovine type IV collagen have amino acid sequences identical or similar to those of human type IV collagen [27].

It is clear from this study that the fraction containing α3-α6(IV)NC1 is nephritogenic. The nephritogenic fraction, P2-P3, contained α1-α6(IV)NC1, and the non-nephritogenic fraction, P2, contained only α1-α2(IV)NC1. Nephritogenicity of NC1 fractions from RBM, LBM, and PBM also supports this view.

An effective purification method for bovine nephritogenic antigen has been reported by Sado et al. [25]. This method relies on antibody-coupled affinity columns for purification. However, the study indicates that antibodies to α3-α6(IV)NC1 should be isolated for use in such a purification system. Purification of the antibodies from antisera of rabbits immunized with bovine RBM-U requires the use of an α1-α2(IV)NC1 fraction containing only a small amount of α3-α6(IV)NC1 as an immunoabsorbent, or even none at all. Sado et al. [22] originally used a fraction from LBM as an absorbent of antibodies to α1-α2(IV)NC1. Although they were able to obtain specific antibodies to α3-α6(IV)NC1, the amount was very small, as LBM is not a good absorbent owing to the considerable amount of α3-α6(IV)NC1 it contains. PBM-U would be an ideal absorbent because, as we have found, it contains a large amount of α1-α2(IV)NC1 and a very small amount of α3-α6(IV)NC1.

Hudson's group has reported that the Goodpasture antigen is the α3(IV)NC1 [3, 4, 8–10, 17, 28, 33–35]. (Goodpasture antigen is defined as the antigen that reacts specifically with antibody in the sera of patients with Goodpasture's syndrome.) Nephritogenic antigen is similarly defined as the antigen that induces glomerulonephritis in animals. Induction of anti-GBM nephritis in rats by the injection of synthetic peptides having part of amino acid sequence of human α3(IV)NC1, α4(IV)NC1, or α5(IV)NC1 [31] supports the view that these three chains are responsible for the induction of the anti-GBM nephritis. In this study, NC1 fractions containing α3(IV)NC1 to α6(IV)NC1 proved to be candidate nephritogenic antigens, but the nephritogenicity of each chain could not be examined.

The nephritogenicity of the α3(IV)NC1 to α6(IV)NC1 could be examined if each chain were separated at a level of high purity, but it seems this would be difficult because the chains bind tightly to each other. Conditions that make the α-chains part from other α-chains are drastic and would destroy almost all structural antigenic determinants [9].

Among the α3(IV) to α6(IV) chains, α6(IV)NC1 does not seem to be responsible for the induction of the anti-GBM nephritis, because the α6(IV) chain is absent from the GBM. This was clearly shown in human GBM by Ninomiya et al. [18] and in bovine GBM in this study. The other three chains, α3(IV), α4(IV), and α5(IV), thus remain as very strong candidates for the function of nephritogenic antigens. The primary structures of these chains deduced by the cDNA technique are very similar [1, 2, 6, 14, 15, 20, 29, 37, 39], and the three chains will assemble and form an α3-α4-α5(IV) helical molecule in human GBM [16, 18]. This assumption is based on the fact that co-absence of α3(IV), α4(IV), and α5(IV) chains in the GBM was observed in the cases of both autosomal recessive and X-linked Alport's syndrome in which the genetic defect was present only in one of the *COL4A3*, *COL4A4*, and *COL4A5* genes [5, 12, 16].

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