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Production of anti-NC1 antibody by affected male dogs with X-linked hereditary nephritis: a probe for assessing the NC1 domain of collagen type IV in dogs and humans with hereditary nephritis

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Summary. Some patients with hereditary nephritis (HN) who have received a renal transplant have been shown to form antibody with specificity for the NC1 domain of collagen type IV, a major constituent of glomerular basement membranes (GBM). We attempted to duplicate this phenomenon in a family of dogs with X-linked HN, a model for human X-linked HN, by immunizing affected male dogs with normal dog NC1 domain. A collagenase digest was prepared from normal dog GBM, the NC1 domain was separated into dimer (~50 kDa) and monomer (24 kDa and 26 kDa) components by SDS-PAGE, and injected into two affected male dogs. Antisera obtained from both dogs contained antibody which reacted with the NC1 domain of dog and human GBM by a plate-binding radioimmunoassay, bound to the dimer and 26 kDa monomer bands by Western blotting, and staining dog and human GBM by immunofluorescence (IF). The affected male dog antiserum reacted equally by radioimmunoassay with the NC1 domain isolated from GBM of unaffected, affected male, and carrier female dogs in the family with X-linked HN, and bound by Western blotting to dimers and the 26 kDa monomer band of the NC1 domain of GBM in each group of dogs. However, the affected male dog antiserum differentiated these dogs by IF; it produced global staining of GBM of unaffected dogs, failed to stain GBM of affected male dogs, and produced segmental staining of GBM of carrier female dogs. Absorption of the affected male dog antiserum with normal dog NC1 domain eliminated the staining of dog GBM by IF, whereas staining persisted after absorption with affected male dog NC1 domain. The abnormal staining patterns of GBM seen by IF in the affected male and carrier female dogs and the results of the absorption studies imply an abnormality of one or more determinants in the 26 kDa monomer band of the NC1 domain of their GBM. Amino acid sequencing of this band identified the α1(IV) chain of collagen type IV, a finding that has implications for the pathogenesis of canine X-linked HN. Absent and segmental staining respectively were also seen by IF in GBM of a male and female patient with HN, using the affected male dog antiserum. Thus, the results obtained in affected male and carrier female dogs with X-linked HN may also be relevant to patients with this disease.

Key words: Canine hereditary nephritis – NC1 domain

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Introduction

Hereditary nephritis (HN) consists of a number of glomerular diseases that differ in various ways, including their mode of inheritance (X-linked, autosomal dominant or, rarely, autosomal recessive; Feingold et al. 1985); the age of onset of symptoms of renal dysfunction; and the rate of deterioration to terminal renal failure (Atkin et al. 1986). Other variables include the presence or absence of extra-renal clinical manifestations, the most common of which are sensorineural hearing loss (the HN is then referred to as Alport's syndrome) and anterior lenticonus (Grünfeld 1985). Renal biopsies obtained from HN patients usually show multilaminar splitting of glomerular basement membranes (GBM) by electron microscopy (Bernstein 1987). Moreover, GBM seen on renal biopsies of most male and some female patients fail to stain by immunofluorescence (IF) using serum obtained from patients with Goodpasture's syndrome (Olson et al. 1980; Jenis et al. 1981; McCoy et al. 1982; Jeraj et al. 1983). Such sera contain antibody to the NC1 domain (Wieslander et al. 1984a, b), which is located at the C-terminal end of the collagen type IV

molecule (Hudson et al. 1989), a major constituent of GBM (Martinez-Hernandes and Amenta 1983).

Most studies of the pathogenesis of HN have been conducted on male patients with the X-linked form of this disease. GBM of these patients have been shown by SDS-PAGE and two-dimensional gel electrophoresis to possess an abnormality of one or more components of the NC1 domain (Kleppel et al. 1987, 1989; Savage et al. 1989). Moreover, after receiving a renal transplant, some patients with HN develop anti-GBM antibody (Querin et al. 1986) with specificity for the NC1 domain (Kashtan et al. 1986; Kleppel et al. 1987, 1989; Savage et al. 1989). In some cases, anti-GBM nephritis occurs in the transplant (Milliner et al. 1982; Fleming et al. 1988; Shah et al. 1988). In this study, we attempted to mimic anti-GBM antibody production in HN patients who have received a renal transplant by injecting normal dog NC1 domain into affected male dogs with X-linked HN. Canine X-linked HN is a spontaneously occurring glomerular disease that closely resembles the X-linked form of human HN in several ways, with rapid development of terminal renal failure in affected male dogs (Jansen et al. 1987), X-linked mode of inheritance (Jansen et al. 1986b), multilaminar splitting of GBM seen by electron microscopy in affected male and carrier female dogs (Jansen et al. 1986a) and failure of GBM of affected male dogs to stain by IF using anti-NC1 antibodies (Thorner et al. 1987, 1989a, c). We found that the immunized affected male dogs produced antibody that was directed against one or more determinants in the 26 kDa monomer band of the NC1 domain, as determined by Western blotting, and stained both normal dog and human GBM by IF. This antibody failed to differentiate the NC1 domain in GBM of unaffected, affected male, and carrier female dogs in the family with X-linked HN by a plate-binding radioimmunoassay or Western blotting. In contrast, by IF the antibody produced distinctive patterns of staining of GBM in the three groups of dogs and in two patients with HN. These studies indicate that there is an abnormality of the NC1 domain in GBM of affected male and carrier female dogs with X-linked HN which involves the 26 kDa monomer band. Amino acid sequencing of this band in dog NCI domain identified the α1(IV) chain of collagen type IV. The above findings have implications regarding the pathogenesis of canine X-linked HN.

Materials and methods

Normal adult mixed-breed dogs were sacrificed following their use for experimental cardiovascular surgery, and their kidneys were removed for preparation of collagenase digests of GBM. In addition, samples of kidney were snap-frozen in liquid nitrogen for examination by IF. Adult carrier female Samoyed dogs in a family with X-linked HN were mated with mixed-breed males (Jansen et al. 1986b), and their offspring underwent renal biopsies at 2–3 months of age. One portion was examined by IF, using an anti-NC1 antibody reactive with normal human and dog NC1 domain in GBM (human anti-NC1 antibody) (Thorner et al. 1989a, c). A second portion was processed for electron microscopy. The IF and electron microscopy studies allowed the offspring to be classified as "unaffected", "affected male", and "carrier female". Dogs were either used for immunization beginning at 3–4 months of age, or sacrificed at 6–8 months, at which time their kidneys were removed and collagenase digests prepared from their GBM. At the time of sacrifice, samples of kidney were snap-frozen in liquid nitrogen for examination of GBM by IF.

The NC1 domain was prepared by collagenase digestion of GBM isolated from kidneys of normal mixed-breed dogs, unaffected, affected male, and carrier female dogs in the family with X-linked HN, and normal human subjects, as described previously (Thorner et al. 1989c), except that following collagenase digestion the NC1 domain was reacted with 20 mM iodoacetamide for 4 h at 4° C, dialysed against 0.02 M phosphate-buffered saline and stored in solution at −20° C. In previous experiments, the product had been precipitated with ethanol and stored as a powder (Thorner et al. 1989c). However, the powder was difficult to redissolve, requiring boiling in SDS. The new method was felt to yield a product closer to the in vivo state of the NC1 domain.

The collagenase digest of GBM prepared from normal mixed-breed dogs was subjected to SDS-PAGE as described previously (Thorner et al. 1989c). Gels were stained with Coomassie brilliant blue and the regions containing the dimer (~50 kDa) and monomer (24 kDa and 26 kDa) components of the NC1 domain were cut out, destained in alcohol, crushed into fragments, and emulsified in incomplete Freund's adjuvant. Two affected male dogs in the family with X-linked HN were injected intramuscularly every 2–3 weeks with 100–200 µg protein of the NC1 domain. Dogs were bled before each injection. The antisera were first enriched for IgG by passing them over a protein-A sepharose column, and eluting the bound fraction with 0.1 M acetic acid. In order to eliminate non-specific binding to non-NC1 domain proteins in kidney, normal mixed-breed dog kidney cortex was sonicated and, following centrifugation, the supernatant was conjugated to cyanogen bromide-activated sepharose (Pharmacia, Uppsala, Sweden). The IgG-enriched fraction of the antisera was then passed over this column and the unbound fraction collected (referred to herein as "kidney-absorbed dog antiserum"). For IF, both intact and kidney-absorbed dog antisera were used, while for radioimmunoassay and Western blotting only kidney-absorbed dog antisera were used, because of the high background seen with the intact antisera. As negative controls, two normal dogs were immunized with the same NC1 domain preparation as used with affected male dogs.

Normal dog NC1 domain was bound to cyanogen bromide-activated sepharose. The kidney-absorbed dog antisera were absorbed with this, and the unbound fraction was tested by radioimmunoassay for reactivity with normal dog NC1 domain. This process was repeated until there was complete loss of reactivity with normal dog NC1 domain. The unbound fraction was then used to stain normal dog kidney by IF. Absorption was also performed using affected male dog NC1 domain conjugated to cyanogen bromide-activated sepharose. The process was repeated until there was complete loss of reactivity with affected dog NC1 domain by radioimmunoassay.

IF was performed as previously described (Thorner et al. 1987), using tissue sections of kidney that were pretreated with either phosphate-buffered saline or acid-urea to expose hidden NC1 domain determinants (Yoshioka et al. 1985b). The human anti-NC1 antibody, intact affected male and normal dog antisera, and the kidney-absorbed dog antisera were used at a 1:3 dilution. The kidney-absorbed dog antisera that had been further absorbed with either normal or affected male dog NC1 domain were used undiluted. All antisera were followed by application of fluoresceinated rabbit anti-human or anti-dog IgG (Cappel, Gathersberg, Md., USA). In the case of the carrier female kidney, double staining was performed using the intact affected male dog antiserum, followed by fluorescein-conjugated anti-dog IgG, and then with a 1:10 dilution of rabbit anti-NC1 antiserum (Thorner et al. 1989a), followed by rhodamine-conjugated anti-rabbit IgG.

A plate-binding radioimmuno assay was performed as previously described (Thorner et al. 1989c). Wells of microtitre plates were coated with dog or human NC1 domain plated in phosphate-buffered saline or acid-urea to expose hidden determinants (Wies-

lander et al. 1985). The coated wells were then reacted with the kidney-absorbed dog antisera or the human anti-NC1 antibody, followed by I^{125} protein A.

The NC1 domain in collagenase digests of GBM obtained from normal humans, normal mixed-breed dogs, and unaffected, affected male, and carrier female dogs in the family with X-linked HN were subjected to Western blotting as previously described (Thorner et al. 1989c). The blots were developed using a 1:50 dilution of the kidney-absorbed dog antisera, or a 1:100 dilution of the human anti-NC1 antibody, followed by I^{125} protein A.

For amino acid sequencing the NC1 domain in collagenase digests of GBM of mixed-breed dogs was separated by SDS-PAGE and electroblotted as previously described (Moos et al. 1988), using Immobilon-P (Millipore) as an inert support. The blot was stained with Coomassie brilliant blue, the individual monomer bands were cut out, and the samples were directly loaded onto a gas phase microsequencer (Porton, model 2090) for amino acid sequencing beginning from the N-terminus.

Renal biopsies obtained from a male and female patient with HN were examined by IF using the affected male dog antiserum. Details of these patients have been previously published (Thorner et al. 1990). Normal kidney resected from a patient with a Wilms' tumour was used as a control.

Results

By radioimmunoassay, antiserum obtained from the affected male dogs reacted equally with normal dog NC1 domain plated in either phosphate-buffered saline or acid-urea (Fig. 1A). In contrast, plating of normal dog NC1 domain in acid-urea resulted in marked enhancement of reactivity with the human anti-NC1 antibody (Fig. 1B). The affected male dog antiserum cross-reacted with human NC1 domain, and no difference in reactivity was seen when the human NC1 domain was plated in phosphate-buffered saline or acid-urea (Fig. 1C).

The molecular weights of normal dog NC1 domain monomers in GBM were determined by comparison with human NC1 domain monomers as seen following SDS-PAGE. The human NC1 domain separated into dimers at about 50 kDa and three monomers at 24 kDa, 26 kDa, and 28 kDa (Fig. 2A), as previously described (Kleppel et al. 1987; Thorner et al. 1989b). The dog NC1 domain separated into dimers at about 50 kDa and two monomer bands that lined up exactly with the 24 kDa and 26 kDa monomer bands of the human NC1 domain (Fig. 2B). In addition, a faint band at 20 kDa was sometimes seen when different preparations of human and dog NC1 domain were examined. We previously reported that the dog NC1 domain separated into dimers and three monomer bands, which were arbitrarily named to correspond with the three monomers of the human NC1 domain, although they did not line up exactly by SDS-PAGE (Thorner et al. 1989c). However, using the modifications for preparation of the NC1 domain (see Materials and methods), it was apparent that the dog NC1 domain did not contain a 28 kDa monomer band and the three bands seen previously were actually at 26 kDa, 24 kDa, and 20 kDa.

Western blotting was used to compare the binding of the human anti-NC1 antibody and affected male dog antisera to normal human and dog NC1 domain. The human anti-NC1 antibody showed strong binding to dimers and the 28 kDa monomer band of the human NC1 domain, with moderate binding to the 26 kDa monomer band, and slight binding to the 24 kDa monomer band (Fig. 2C). The human anti-NC1 antibody bound to dimers and the 26 kDa and 24 kDa monomer bands of the dog NC1 domain (Fig. 2D). In

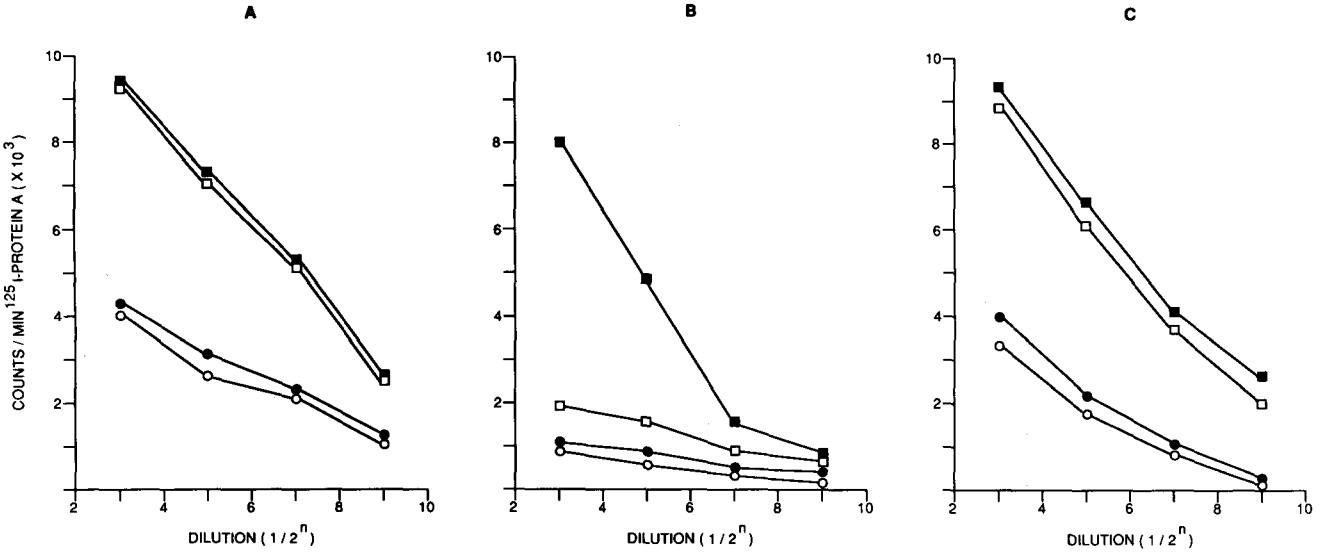

Fig. 1. Plate-binding radioimmunoassay, showing reactivity of affected male dog antiserum (**A, C**), and human anti-NC1 antibody (**B**), with dog (**A, B**) and human (**C**) NC1 domain. Wells of microtitre plates were coated with the NC1 domain plated in either phosphate-buffered saline or acid-urea. Pre-immune dog serum and plasmapheresis fluid obtained from a patient treated for hypercholesterolemia served as negative controls. Reactivity wa assessed by binding of I^{125} protein A. **A** Antigen: dog NC1 in acid-urea (■, ●), in phosphate-buffered saline (□, ○); antibody: dog anti-dog NC1 (■, □); control: normal dog serum (●, ○). **B** Antigen: dog NC1 in acid-urea (■, ●), in phosphate-buffered saline (□, ○); antibody: human anti-NC1 (■, □); control: plasmapheresis fluid (●, ○). **C** Antigen: human NC1 in acid-urea (■, ●), in phosphate-buffered saline (□, ○); antibody: dog anti-dog NC1 (■, □); control: normal dog serum (●, ○)

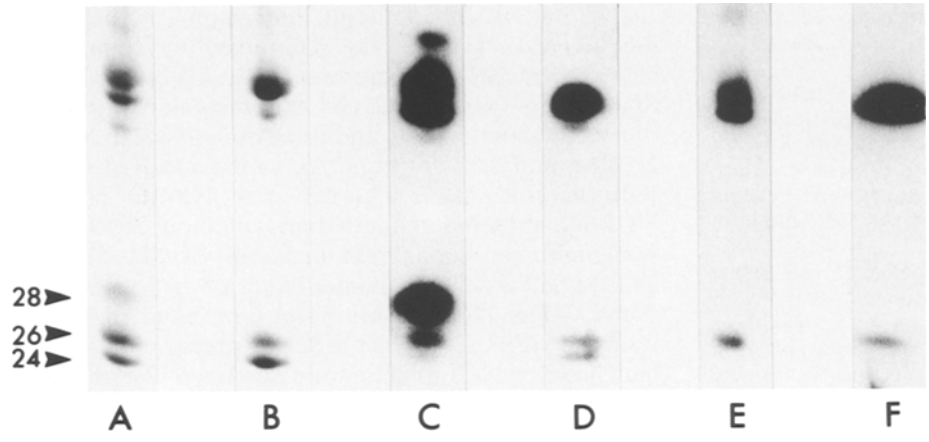

Fig. 2. SDS-PAGE of normal human (**A**) and dog (**B**) NC1 domain and Western blotting of human (**C, E**) and dot (**D, F**) NC1 domain in glomerular basement membrane (GMB). The NC1 domain was separated by SDS-PAGE, transferred to nitrocellulose, and treated with human anti-NC1 antibody (**C, D**) or affected male dog antiserum (**E, F**) followed by I^{125} protein A. The *arrowheads* at the left mark the positions of the 24 kDa, 26 kDa, and 28 kDa human NC1 domain monomer bands

the case of the affected male dog antiserum, binding to dimers and the 26 kDa monomer band of the human (Fig. 2E) and dog NC1 domains (Fig. 2F) was observed. No 28 kDa monomer band in the dog NC1 domain was detected by Western blotting, using either the human anti-NC1 antibody or the affected male dog antiserum. Similarly, no binding of these reagents to the 20 kDa band of either the human or dog NC1 domain preparations was seen. This result, plus the fact that the presence of the 20 kDa band was variable in both the human and dog NC1 domain preparations, led to the conclusion that this band was either a degradation product or was not part of the NC1 domain.

By IF, antiserum obtained from the affected male dogs produced linear staining of all GBM (i.e. global staining) of normal mixed-breed dogs and normal humans, but only after treatment of tissue sections with acid-urea (Fig. 3). This result was similar to that previously found using the human anti-NC1 antibody (Thorner et al. 1989a, c). No staining of GBM was observed using serum obtained from the immunized normal dogs (data not shown).

By radioimmunoassay, the affected male dog antiserum reacted equally with the NC1 domain in GBM of unaffected (Fig. 4A), affected male (Fig. 4B), and carrier female (Fig. 4C) dogs. No difference was seen when the NC1 domain was plated in either phosphate-buffered saline or acid-urea. Similarly, equal reactivity of the human anti-NC1 antibody was seen with these three groups of dogs (results not shown).

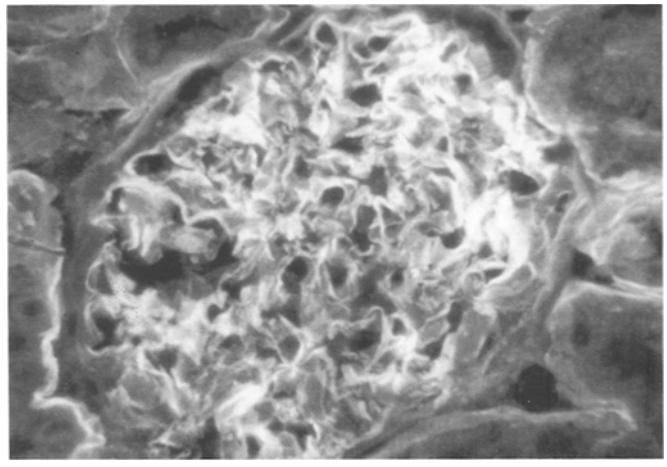

Fig. 3. Immunofluorescence of normal dog kidney using antiserum obtained from an affected male dog, showing global staining of GBM. ×400

By Western blotting, the affected male dog antiserum bound to dimers and the 26 kDa monomer band of the NC1 domain of all three groups of dogs (Fig. 5). The human anti-NC1 antibody bound to dimers and both

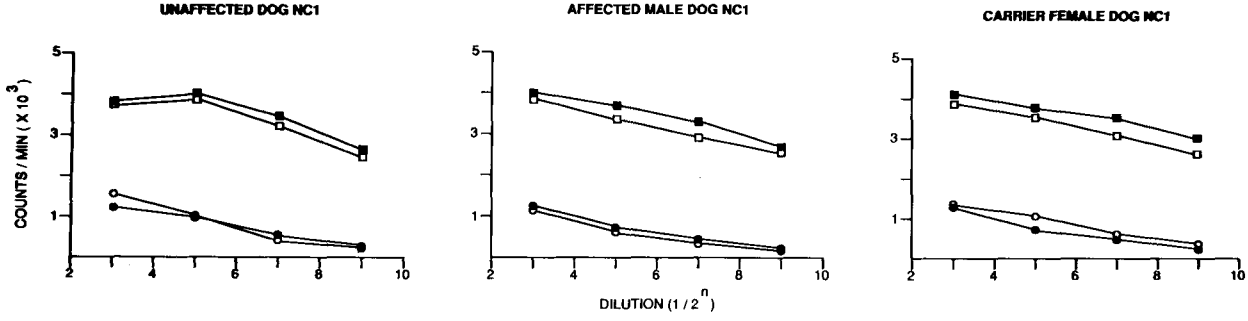

Fig. 4. Plate-binding radioimmunoassay showing reactivity of affected male dog antiserum with the NC1 domain in GBM of unaffected, affected male and carrier female dogs. Wells of microtitre plates were coated with the NC1 domain plated in either phosphate-buffered saline (PBS) or acid-urea. Pre-immune dog serum served as a negative control. Reactivity was assessed by binding of I^{125} protein A. □, NC1 in PBS vs affected male dog antiserum; ■, NC1 in acid-urea vs affected male dog antiserum; ○, NC1 in PBS vs normal dog serum; ●, NC1 in acid-urea vs normal dog serum

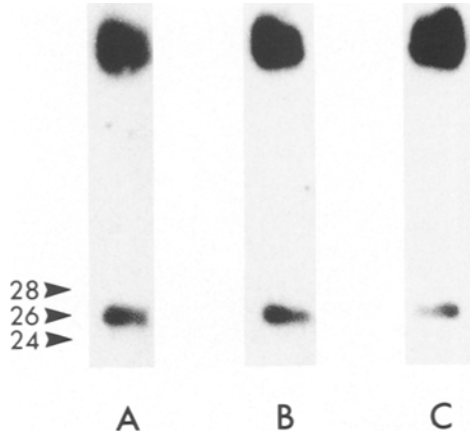

Fig. 5. Western blotting of the NC1 domain in GBM of (**A**) unaffected, (**B**) affected male, and (**C**) carrier female dogs in the family with X-linked hereditary nephritis. The NC1 domain was separated by SDS-PAGE, transferred to nitrocellulose, and treated with affected male dog antiserum, followed by I^{125} protein A. The *arrowheads* at the left mark the positions of the 24 kDa, 26 kDa, and 28 kDa human NC1 domain monomer bands

the 24 kDa and 26 kDa monomer bands; no difference was seen between any of the three groups of dogs (results not shown). Previously, we had reported that this antibody showed decreased reactivity with the NC1 domain of affected male dogs by radioimmunoassay, and failed to bind to the 24 kDa and 26 kDa monomer bands by Western blotting (Thorner et al. 1989c). However, when the NC1 domain was prepared by the modified method used in this study, the human anti-NC1 antibody failed to distinguish unaffected, affected male, and carrier female dogs by radioimmunoassay or Western blotting.

By IF, using the affected male dog antiserum, no staining of GBM was observed if tissue sections of kidney were not treated with acid-urea. However, after acid-urea treatment, this antiserum produced linear staining of all GBM of unaffected dogs (Fig. 6A), failed to stain GBM of affected male dogs (Fig. 6B), and produced linear staining of some but not all GBM (i.e. segmental staining) of carrier female dogs (Fig. 6C, D). The antiserum cross-reacted by IF with GBM of normal humans (Fig. 7A), failed to stain GBM of a male patient with HN (Fig. 7B), and produced segmental staining of GBM

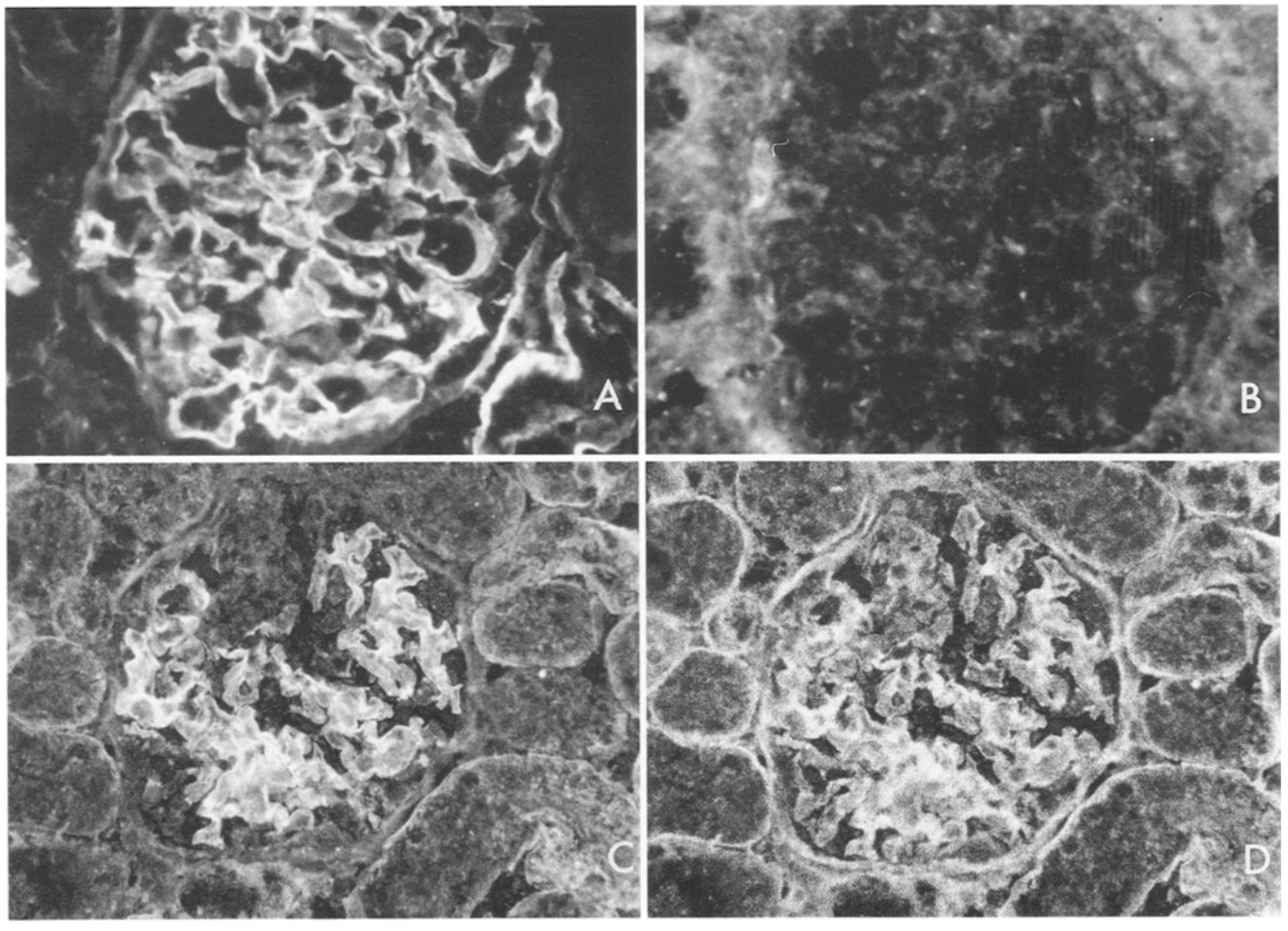

Fig. 6. Immunofluorescence of kidneys of Samoyed dogs in the family with X-linked hereditary nephritis using affected male dog antiserum (**A–C**) and rabbit anti-NC1 antiserum (**D**). **A** Glomerulus of unaffected dog showing global staining of GBM; **B** glomerulus of affected male dog showing absence of staining of GBM; **C** glomerulus of carrier female dog showing segmental staining of GBM; and **D** same glomerulus of carrier female dog showing global staining of GBM. ×400

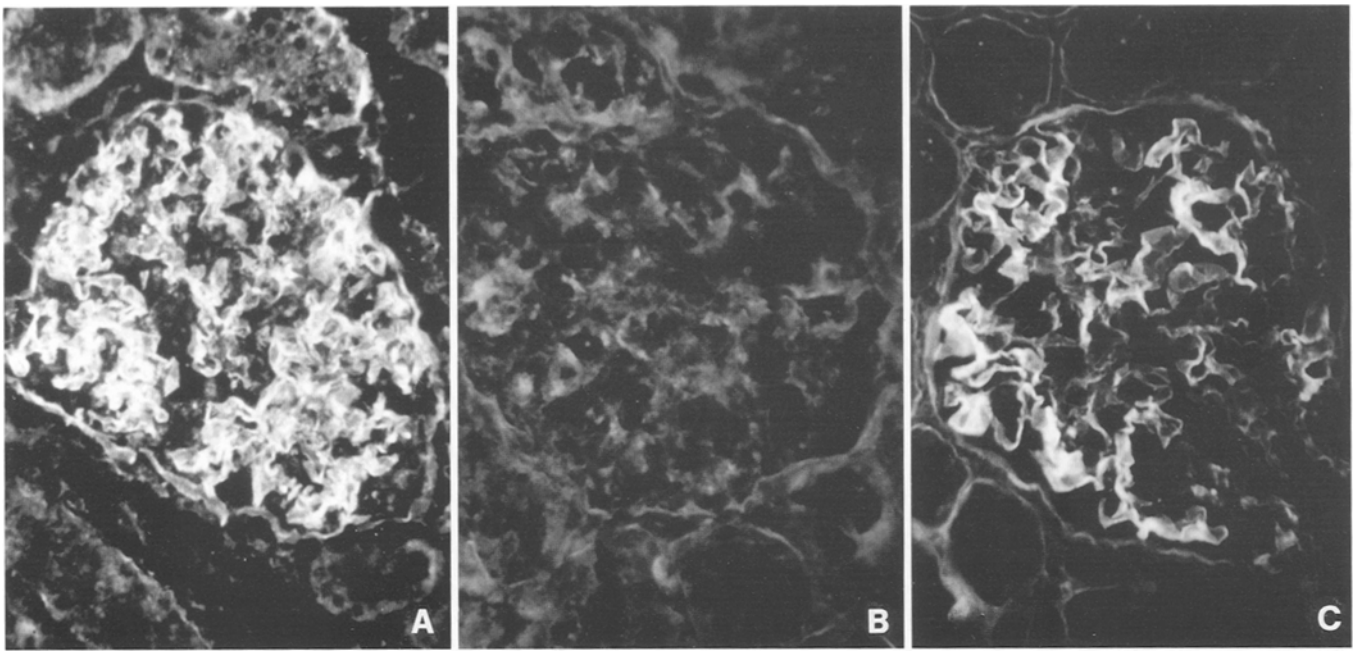

Fig. 7. Immunofluorescence of kidneys of a normal human and two patients with hereditary nephritis (HN) using affected male dog antiserum: **A** glomerulus of normal human showing global staining of GBM; **B** glomerulus of a male patient with HN showing absence of staining of GBM, **C** glomerulus of a female patient with HN showing segmental staining of GBM. ×400

of a female patient with HN (Fig. 7C). These results are identical to those obtained previously in the family of dogs with X-linked HN (Thorner et al. 1989a, c) and the same two patients with HN (Thorner et al. 1990) using the human anti-NC1 antibody.

The kidney-absorbed dog antiserum that had been further absorbed with normal dog NC1 domain failed to stain GBM by IF using sections of normal dog kidney. In contrast, kidney-absorbed dog antiserum that had been further absorbed with affected male dog NC1 domain continued to show positive staining of GBM, similar to that seen in Fig. 3.

Amino terminal sequencing of the 24 kDa monomer band of normal dog NC1 domain yielded the 21 amino acid sequence: GMPGRSVSIGYLLVKHIQTDQ. This differs by one amino acid only (i.e. 95% homology) from the human α2(IV) chain sequence: GMPGRSVSIGYLLVKHSQTDQ, which is present at the junction of the triple helix and the NC1 domain (Hostikka and Tryggvason 1988). Sequencing of the 26 kDa monomer band of normal dog NC1 domain yielded the 23 amino acid sequence: GPPGTPSVDHGFLVTRHSQTNDD. This differs by one amino acid only, (96% homology) from the human α1(IV) chain sequence: GPPGTPSVDHGFLVTRHSQTIDD, which is present at the junction of the triple helix and the NC1 domain (Pihlajaniemi et al. 1985). No sequence data for the 20 kDa band were obtainable; thus its origin could not be determined.

Discussion

The collagen type IV molecule exists as a triple helix of α chains, and is joined to a second collagen type IV molecule by the NC1 domain, which is located at the C-terminal end (Hudson et al. 1989). This domain can be separated from the rest of collagen type IV by collagenase digestion, producing a hexamer consisting of the C-terminal regions of 6 α chains. When examined by SDS-PAGE, normal human NC1 domain can be separated into three monomers of 24 kDa, 26 kDa, and 28 kDa, and dimers of these monomers of about 50 kDa (Kleppel et al. 1987). The 24 kDa and 26 kDa monomers are derived from the α2(IV) and α1(IV) chains of collagen type IV respectively (Butkowski et al. 1987, 1990). The 28 kDa monomer can be separated by two-dimensional gel electrophoresis into strongly (28+++ kDa) and weakly (28+ kDa) cationic forms (Yoshioka et al. 1985a), which are derived from the recently described α3(IV) and α4(IV) chains of collagen type IV respectively (Langeveld et al. 1988; Saus et al. 1988; Butkowski et al. 1990). Western blotting of normal human NC1 domain, using sera obtained from patients with Goodpasture's syndrome, has shown major binding to the 28 kDa monomer, particularly to the 28+++ kDa form, and lesser binding to other monomers (Yoshioka et al. 1985a; Butkowski et al. 1990). Recently, a fifth chain of collagen type IV has been described, the α5(IV) chain (Hostikka et al. 1990). Its NC1 domain shows marked homology to that of the α1(IV) chain, and both have a molecular weight of 26 kDa. The mRNA for the α5(IV) chain comprises only a minor component (<2%) of the total mRNA for the collagen type IV present in kidney. There is evidence that the α1(IV) and α2(IV) chains form one type of collagen type IV network, while the other three chains form a second type of collagen type IV network (Kleppel et al. 1992).

Human X-linked HN is believed to be the result of

an abnormality in the NC1 domain. This view is based on IF studies showing that GBM of most male and some female patients fail to stain using sera obtained from patients with Goodpasture's syndrome (Olson et al. 1980; Jenis et al. 1981; McCoy et al. 1982; Jeraj et al. 1983), which contain anti-NC1 antibody (Wieslander et al. 1984a, b). Moreover, the 28 kDa monomer of the NC1 domain could not be detected in GBM of some patients with X-linked HN by SDS-PAGE, two dimensional gel electrophoresis, and Western blotting using serum obtained from patients with Goodpasture's syndrome (Kleppel et al. 1987; Savage et al. 1989). However, some investigators have shown that the 26 kDa monomer of the NC1 domain was abnormal in GBM of some patients with X-linked HN, since it failed to bind by Western blotting to sera obtained from patients with Goodpasture's syndrome (Savage et al. 1989). Hence, an abnormality of this component of the NC1 domain could be the primary defect in X-linked HN. Support for the latter view has come from the observation that some patients with HN who have received a renal transplant developed anti-GBM antibody, implying exposure to novel antigens (Querin et al. 1986). By Western blotting, sera obtained from most of these patients bound to the 26 kDa monomer of normal human NC1 domain (Kashtan et al. 1986). The loss of the 28 kDa monomer of the NC1 domain from GBM could then be a secondary abnormality (Kleppel et al. 1987, 1989b); Savage et al. 1989; Thorner et al. 1989b). This hypothesis is supported by a report that a component of the 26 kDa monomer band forms a dimer with the 28 kDa monomer, and that this component is not derived from the α1(IV) chain (Kleppel et al. 1992). Since the NC1 domain of the α5(IV) chain also has a molecular weight of 26 kDa (Hostikka et al. 1990), and mutations in the coding region for the NC1 domain of the α5 chain of collagen type IV have recently been identified at the gene level in three families with X-linked HN (Barker et al. 1990; Zhou et al. 1991), a primary defect in the α5(IV) chain could conceivably result in secondary loss of the 28 kDa monomer.

Canine X-linked HN has been found to be a model for human X-linked HN, since it shares many similarities at the clinical, genetic, morphological, and immunohistochemical levels (Jansen et al. 1986a, b; Thorner et al. 1987, 1989a, c). Hence, we attempted to mimic the situation in which patients with HN are exposed to novel antigens in a renal transplant by injecting normal dog NC1 domain monomers and dimers into affected male dogs with X-linked HN. The affected male dog antiserum bound to normal dog NC1 domain by radioimmunoassay and Western blotting and produced positive staining of normal dog GBM by IF. Binding to both dimer and monomer bands was seen, consistent with the fact that the NC1 domain dimers are derived from the monomers, and would share some of the same epitopes. Absorption of the affected male dog antiserum with normal dog NC1 domain removed the staining of normal dog GBM by IF, whereas absorption with affected male dog NC1 domain did not, implying that the latter lacked one or more NC1 domain determinants.

Although the experiment of injecting denatured normal dog NC1 domain into affected male dogs with X-linked HN may not be comparable to kidney transplantation in HN patients, injected normal dogs did not make antibody and the affected male dog antiserum did show similarities to serum obtained from HN patients who received a renal transplant. By IF both failed to stain GBM of male patients with X-linked HN and produced segmental staining of GBM of female patients (Kleppel et al. 1989; Thorner et al. 1990). Both bound to the 26 kDa monomer of human NC1 domain by Western blotting (Kleppel et al. 1989). In this regard, these reagents differ from human anti-GBM antibodies in sera obtained from patients with Goodpasture syndrome, which bind mainly to the 28 kDa monomer of human NC1 domain (Kleppel et al. 1987, 1989; Savage et al. 1989), as did the human anti-NC1 antibody used in our study.

Both the affected male dog antiserum and the human anti-NC1 antibody distinguished unaffected, affected male, and carrier female dogs in the family with X-linked HN by producing distinctive patterns of staining of GBM by IF (Thorner et al. 1989a, c). This result would seem to contradict the radioimmunoassay and Western blotting data obtained using these two reagents, which showed equivalent reactivity with the NC1 domain of GBM in these three groups of dogs. We postulate that both the dog and human anti-NC1 antibodies interact with determinants that are present in the NC1 domain of all dogs, but are abnormally hidden or improperly formed in vivo in affected male and carrier female dogs. These determinants are inaccessible in kidney tissue sections of these dogs, even after denaturation with acid-urea, which helps to expose hidden epitopes (Wieslander et al. 1985; Yoshioka et al. 1985b). The result is that the abnormal GBM in these dogs fail to stain by IF. However, once the NC1 domain is released from the remainder of the collagen molecule by collagenase digestion, it is no longer under the folding constraints dictated by the triple helical portion; hence, these same determinants either become accessible or spontaneously reform, with the result that the reactivity of the NC1 domain of affected male and carrier female dogs, seen both by radioimmunoassay and Western blotting, is equivalent to that seen in unaffected dogs.

In order to interpret our findings using the dog and human anti-NC1 antibodies at the molecular level, amino acid sequencing was performed on each monomer band of normal dog NC1 domain. Just as with the human NC1 domain, the 24 kDa and 26 kDa monomer bands of the dog NC1 domain were found to be derived from the α2(IV) and α1(IV) chains respectively. No α3(IV), α4(IV) or α5(IV) chains were identified, although the sequencing technique we used would not be sensitive enough to detect these chains if they constitute only a minor component of these bands. Thus, it remains undetermined whether the α3(IV), α4(IV) or α5(IV) chains of collagen type IV are present in dog GBM and, if so, which monomer bands contain their NC1 domains. Our sequencing data lead us to conclude that the affected male dog antiserum, which binds to the 26 kDa

monomer band of normal dog NC1 domain, contains antibody to the α1(IV) chain, and that the human anti-NC1 antibody, which binds to the 26 kDa and 24 kDa monomer bands, contains antibody to the the α1(IV) and α2(IV) chains of the dog NC1 domain. However, it remains a possibility that the human anti-NC1 antibody reacts with the α3(IV) and/or α4(IV) chains, should their monomers in the dog NC1 domain migrate to 24 kDa or 26 kDa, since the major reactivity of this antibody with the human NC1 domain is with the 28 kDa monomer band, which is derived from the α3(IV) and α4(IV) chains (Langeveld et al. 1988; Saus et al. 1988; Butkowski et al. 1990). Furthermore, either antibody could theoretically react with the α5(IV) chain, even though the α5(IV) monomer of dog NC1 domain was not detected biochemically.

Assuming that the affected male dog antiserum is directed against the α1(IV) chain and the human anti-NC1 antibody against the α1(IV) and α2(IV) chains of the dog NC1 domain, our IF results imply that both of these chains are abnormal in canine X-linked HN. However, the genes coding for the α1(IV) and α2(IV) chains of collagen type IV are autosomal in man (Emanuel et al. 1986; Griffin et al. 1987), and almost certainly in dogs as well (Ohno's hypothesis). Positing either of these genes as the site of the primary defect in HN would be at variance with the well-established X-linked genetics of inheritance of HN seen in most human families (Feingold et al. 1985) and in the Samoyed dog pedigree (Jansen et al. 1987), and with the identification of mutations in the X-linked gene for the α5(IV) chain in three families with X-linked HN (Barker et al. 1990; Zhou et al. 1991). We believe that the negatively stained GBM seen by IF in affected male and carrier female dogs with X-linked HN reflect abnormalities in the α1(IV) and α2(IV) chains, but we postulate that these abnormalities are secondary to a primary defect involving the α5(IV) chain, leading to improper assembly and/or folding of the α1(IV) and α2(IV) chains. This hypothesis would account for the observation that the immunological abnormalities seen in GBM in this disease are detected only in the in vivo state (i.e. by IF using kidney tissue sections). The NC1 domains of both the α1(IV) and α2(IV) chains would be predicted to have normal sequences, so that once these domains are freed from the rest of the collagen molecule by collagenase digestion, no abnormalities would be detected by radioimmunoassay or Western blotting. A similar situation might apply to humans with X-linked HN. Confirmation of this hypothesis in canine and human X-linked HN will require a study of the α5(IV) chain at the gene, message and protein levels.

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References

Atkin CL, Gregory MC, Broder WA (1986) Alport syndrome. In: Schier RW, Gottschalk CW (eds) Strauss and Welt's diseases of the kidney, 4th edn. Little Brown, Boston, pp 617–641

Barker DF, Hostikka SL, Zhou J, Chow LT, Oliphant AR, Gerken SC, Gregory MC, Skolnick MH, Atkin CL, Tryggvason K (1990) Identification of mutations in the COL4A5 collagen gene in Alport syndrome. Science 248:1224–1227

Bernstein J (1987) The glomerular basement membrane abnormality in Alport's syndrome. Am J Kidney Dis 10:222–229

Butkowski RJ, Langeveld JP, Wieslander J, Hamilton J, Hudson BG (1987) Loalization of the Goodpasture epitope to a novel chain of basement membrane collagen. J Biol Chem 262:7874–7877

Butkowski RJ, Shen G-Q, Wieslander J, Michael AF, Fish AJ (1990) Characterization of type IV collagen NC1 monomers and Goodpasture antigen in human renal basement membranes. J Lab Clin Med 115:365–373

Emanuel BS, Sellinger BT, Gudas LJ, Myers JC (1986) Localization of the human procollagen α1(IV) gene to chromosome 13q34 by in situ hybridization. Am J Hum Genet 38:38–44

Feingold J, Bois E, Chompret A, Broyer M, Gubler M-C, Grünfeld J-P (1985) Genetic heterogeneity of Alport syndrome. Kidney Int 27:672–677

Fleming SJ, Savage CO, McWilliam LJ, Pickering SJ, Ralston AJ, Johnson RW, Ackrill P (1988) Anti-glomerular basement membrane antibody-mediated nephritis complicating transplantation in a patient with Alport's syndrome. Transplantation 46:857–859

Griffin CA, Emanuel BS, Hansen JR, Cavenee WK, Myers JC (1987) Human collagen genes encoding basement membrane α1(IV) and α2(IV) chains map to the distal long arm of chromosome 13. Proc Natl Acad Sci USA 84:512–516

Grünfeld J-P (1985) The clinical spectrum of hereditary nephritis. Kidney Int 27:83–92

Hostikka SL, Tryggvason K (1988) The complete primary structure of the α2 chain of human type IV collagen and comparison with the α1(IV) chain. J Biol Chem 263:19488–19493

Hostikka SL, Eddy RL, Byers MG, Hoyhtya, Shows TB, Tryggvason K (1990) Identification of a distinct type IV collagen α chain with restricted kidney distribution and assignment of its gene to the locus of X chromosome-linked Alport syndrome. Proc Natl Acad Sci USA 87:1606–1610

Hudson BG, Wieslander J, Wisdom BJ Jr, Noelken ME (1989) Biology of disease. Goodpasture syndrome: molecular architecture and function of basement membrane antigen. Lab Invest 61:256–269

Jansen B, Thorner P, Baumal R, Valli V, Maxie MG, Singh A (1986a) Samoyed hereditary glomerulopathy (SHG). Evolution of splitting of glomerular capillary basement membranes. Am J Pathol 125:536–545

Jansen B, Tryphonas L, Wong J, Thorner P, Maxie MG, Valli VE, Baumal R, Basrur PK (1986b) Mode of inheritance of Samoyed hereditary glomerulopathy: an animal model of hereditary nephritis in humans. J Lab Clin Med 107:551–555

Jansen B, Valli VE, Thorner P, Baumal R, Lumsden JH (1987) Samoyed hereditary glomerulopathy: serial, clinical and laboratory (urine, serum biochemistry and hematology) studies. Can J Vet Res 51:387–393

Jenis EH, Valeski JE, Calcagno PL (1981) Variability of anti-GBM binding in hereditary nephritis. Clin Nephrol 15:111–114

Jeraj K, Kim Y, Vernier RL, Fish AJ, Michael AF (1983) Absence of Goodpasture's antigen in male patients with familial nephritis. Am J Kidney Dis 2:626–629

Kashtan C, Fish AJ, Kleppel M, Yoshioka K, Michael AF (1986) Nephritogenic antigen determinants in epidermal and renal basement membranes of kindreds with Alport-type familial nephritis. J Clin Invest 78:1035–1044

Kleppel MM, Kashtan CE, Butkowski RJ, Fish AJ, Michael AF (1987) Alport familial nephritis: absence of 28 kilodalton non-

collagenous monomers of type IV collagen in glomerular basement membrane. J Clin Invest 80:263–266

Kleppel MM, Kashtan C, Santi PA, Wieslander J, Michael AF (1989) Distribution of familial nephritis antigen in normal tissue and renal basement membranes of patients with homozygous and heterozygous Alport familial nephritis. Relationship of familial nephritis and Goodpasture antigens to novel collagen chains and type IV collagen. Lab Invest 61:278–289

Kleppel MM, Fan WW, Cheong HI, Michael AF (1992) Evidence for separate networks of classical and novel basement membrane collagen. Characterization of α3(IV)-Alport antigen heterodimer. J Biol Chem 267:4137–4142

Langeveld JPM, Wieslander J, Timoneda J, McKinney P, Butkowski RJ, Wisdom BJ Jr, Hudson BG (1988) Structural heterogeneity of the noncollagenous domain of basement membrane collagen. J Biol Chem 263:10481–10488

Martinez-Hernandez A, Amenta PS (1983) The basement membrane in pathology. Lab Invest 48:656–677

McCoy RC, Johnson HK, Stone WJ, Wilson CB (1982) Absence of nephritogenic GBM antigen(s) in some patients with hereditary nephritis. Kidney Int 21:642–652

Milliner DS, Pierides AM, Holley KE (1982) Renal transplantation in Alport's syndrome. Anti-glomerular basement membrane glomerulonephritis in the allograft. Mayo Clin Proc 57:35–43

Moos M Jr, Nguyen NY, Liu T-Y (1988) Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. J Biol Chem 263:6005–6008

Olson DL, Anand SK, Landing BH, Heuser E, Grushkin CM, Lieberman E (1980) Diagnosis of hereditary nephritis by failure of glomeruli to bind anti-glomerular basement membrane antibodies. J Pediatr 96:697–699

Pihlajaniemi T, Tryggvason K, Myers JC, Kurkinen M, Lebo R, Cheung M-C, Prockop DJ, Boyd CD (1985) cDNA clones coding for the pro-α1(IV) chain of human type IV procollagen reveal and unusual homology of amino acid sequences in two halves of the carboxyl-terminal domain. J Biol Chem 260:7681–7687

Quérin S, Noël L-H, Grünfeld J-P, Droz D, Mahieu P, Berger J, Kreis H (1986) Linear glomerular IgG fixation in renal allografts: incidence and significance in Alport's syndrome. Clin Nephrol 25:134–140

Saus J, Wieslander J, Langeveld JP, Quinones S, Hudson BG (1988) Identification of the Goodpasture antigen as the alpha 3(IV) chain of collagen IV. J Biol Chem 263:13374–13380

Savage COS, Noel L-H, Crutcher E, Price SRG, Grunfeld JP, Lockwood CM (1989) Hereditary nephritis: immunoblotting studies of the glomerular basement membrane. Lab Invest 60:613–618

Shah B, First MR, Mendoza NC, Clyne DH, Alexander JW, Weiss MA (1988) Alport's syndrome: risk of glomerulonephritis induced by anti-glomerular-basement-membrane antibody after renal transplantation. Nephron 50:34–38

Thorner P, Jansen B, Baumal R, Valli VE, Goldberger A (1987) Samoyed hereditary glomerulopathy. Immunohistochemical staining of basement membrane sof kidney for laminin, collagen type IV, fibronectin, and Goodpasture antigen, and correlation with electron microscopy of glomerular capillary basement membranes. Lab Invest 56:435–443

Thorner P, Baumal R, Binnington A, Valli VEO, Marrano P, Clarke H (1989a) The NC1 domain of collagen type IV in neonatal dog glomerular basement membranes. Significance in Samoyed hereditary glomerulopathy. Am J Pathol 134:1047–1054

Thorner PS, Baumal R, Eddy A, Marrano P (1989b) Characterization of the NC1 domain of collagen type IV in glomerular basement membranes (GBM) and of antibodies to GBM in a patient with anti-GBM nephritis. Clin Nephrol 31:160–168

Thorner P, Baumal R, Valli VEO, Mahuran D, McInnes R, Marrano P (1989c) Abnormalities in the NC1 domain of collagen type IV in GBM in canine hereditary nephritis. Kidney Int 35:843–850

Thorner PS, Baumal R, Eddy A, Marrano PM (1990) A study by immunofluorescence microscopy of the NC1 domain of collagen type IV in glomerular basement membranes of two patients with hereditary nephritis. Virchows Arch [A] 416:205–212

Wieslander J, Barr JF, Butkowski RJ, Edwards SJ, Bygren P, Heinegård D, Hudson BG (1984a) Goodpasture antigen of the glomerular basement membrane: localization to noncollagenous regions of type IV collagen. Proc Natl Acad Sci USA 81:3838–3842

Wieslander J, Bygren P, Heinegård D (1984b) Isolation of the specific glomerular basement membrane antigen involved in Goodpasture syndrome. Proc Natl Acad Sci USA 81:1544–1548

Wieslander J, Langeveld J, Butkowski R, Jodlowski M, Noelken M, Hudson BG (1985) Physical and immunochemical studies of the globular domain of type IV collagen: cryptic properties of the Goodpasture antigen. J Biol Chem 260:8564–8570

Yoshioka K, Kleppel M, Fish AJ (1985a) Analysis of nephritogenic antigens in human glomerular basement membrane by two-dimensional gel electrophoresis. J Immunol 134:3831–3837

Yoshioka K, Michael AF, Velosa J, Fish AJ (1985b) Detection of hidden nephritogenic antigen determinants in human renal and nonrenal basement membranes. Am J Pathol 121:156–165

Zhou J, Barker DF, Hostikka SL, Gregory MC, Atkin CL, Tryggvason K (1991) Single base mutation in α5(IV) collagen chain gene converting a conserved cysteine to serine in Alport syndrome. Genomics 91:10–18