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A study by immunofluorescence microscopy of the NC1 domain of collagen type IV in glomerular basement membranes of two patients with hereditary nephritis

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Summary. The NC1 domain of the collagen type IV molecule, the major component of glomerular basement membranes (GBM), consists of dimers and 24 kilodalton (K), 26 K and 28 K monomers in man, and contains the Goodpasture antigen. Serum obtained from patients with Goodpasture's syndrome has been reported not to stain GBM of most male and some female patients with hereditary nephritis (HN) by immunofluorescence (IF) microscopy. In the present study, GBM seen on the renal biopsies of 2 patients (one male and one female) with HN were examined by IF to ascertain whether NC1 monomers were detectable. Three reagents were used: a plasmapheresis fluid (PPF) obtained from a patient who was treated for anti-GBM nephritis (human anti-GBM PPF); a commercial rabbit antibody against human NC1; and a rabbit antibody raised by us against dog NC1, which cross-reacted with human NC1. All 3 reagents detected NC1 determinants in GBM of normal human kidney by IF and reacted with human NC1 by a plate-binding radioimmunoassay (RIA). The human anti-GBM PPF bound to 28 K and 26 K monomer components of NC1 by Western blotting, the rabbit anti-human NC1 antibody bound to 26 K and 24 K monomers, while the rabbit anti-dog NC1 antibody bound only to the 26 K monomer. By IF, the human anti-GBM PPF did not stain GBM of the male patient with HN, but produced segmental staining of GBM (i.e., some GBM stained, while others did not) of the female patient. In contrast, the rabbit anti-NC1 antibodies produced global staining by IF of GBM of both patients. The absence of staining (i.e., global or segmental) seen with the human anti-GBM PPF implied that the 26 K and 28 K monomers of NC1 were either absent from GBM, or were present but altered structurally, leading to a diminution in their immunological reactivity. However, the positive staining observed with the rabbit anti-NC1 antibodies implied that the 26 K monomer was actually present in GBM. Hence, we postulate that the 26 K monomer of NC1 in GBM was structurally altered, and that the 28 K monomer was either absent, or present but altered. These findings suggest that there is an abnormality of more than one monomer of NC1 in GBM of patients with HN.

Key words: Goodpasture antigen – NC1 domain – Hereditary nephritis – Alport's syndrome – collagen type IV

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Introduction

Human hereditary nephritis (HN) consists of a group of inherited glomerular diseases and is often associated with sensorineural hearing loss, referred to as Alport's syndrome, and lens abnormalities, particularly anterior lenticonus (Habib et al. 1982; Grünfeld 1985). Most patients present with haematuria, either microscopic or gross, and eventually develop proteinuria. Males usually progress to end stage renal failure by the end of the third decade, whereas females follow a more variable course, ranging from no clinical abnormalities to end stage renal disease (Grünfeld 1985; Habib et al. 1982). HN can be grouped into subtypes based on the age at which end stage renal failure occurs, the presence of extra-renal manifestations, and the mode of inheritance, either X-linked or autosomal, as determined by pedigree analysis (Atkins et al. 1986; Feingold et al. 1985). A diagnosis of HN can be made with certainty only by examination

of glomeruli using electron microscopy (EM) which shows multilaminar splitting of glomerular basement membranes (GBM) (Grünfeld 1985; Habib et al. 1982; Spear and Slusser 1972). It has been suggested that, in HN, there is an abnormality of Goodpasture antigen (GPA) (Kleppel et al. 1987, Savage et al. 1989), which is contained in the NC1 domain at the C-terminus of the collagen type IV molecule (Wieslander et al. 1985; Butkowski et al. 1985, 1987; Kleppel et al. 1986a). The NC1 domain is composed of dimers and monomers of molecular weights 24 K, 26 K and 28 K (Weislander et al. 1985, Butkowski et al. 1985, Kleppel et al. 1986) and cross-links collagen type IV molecules in the supramolecular formation of GBM (Bächinger et al. 1982; Wieslander et al. 1984a; Weber et al. 1984). Hence a defect in it could result in weakened GBM and the splitting seen in HN.

In the present study, we assessed whether the 24 K, 26 K and 28 K monomers of the NC1 domain of the collagen type IV molecule could be detected by immunofluorescence (IF) in GBM seen on the renal biopsies of two patients with HN, one male and one female. We used a plasmapheresis fluid (PPF) obtained from a patient who was treated for anti-GBM nephritis, which contained antibodies to the 26 K and 28 K monomers of NC1, and 2 rabbit antisera against NC1, which contained antibodies only to the 24 K and 26 K monomers. The PPF did not stain GBM of the male patient with HN but produced segmental staining of GBM (i.e. some GBM stained while others did not) of the female patient. In contrast, the rabbit anti-NC1 antibodies produced global staining of GBM of both patients. These findings indicated that the 26 K monomer was present but altered structurally, leading to a diminution in its immunological reactivity, and that the 28K monomer was either absent or present but altered. These results have allowed us to speculate on the pathogenesis of human HN.

Materials and methods

Two patients who underwent renal biopsies at The Hospital for Sick Children, Toronto and were subsequently diagnosed as having HN form the basis of the present study.

The renal biopsies were divided into 3 portions. The first was fixed in 10% buffered formalin and processed for light microscopy (LM). The second was snap-frozen in liquid nitrogen; sections were stained by direct IF, using fluorescein-conjugated rabbit antisera to human immunoglobulins (IgG, IgA, and IgM) and C3 (Meloy, Springfield, Virginia), and by indirect IF (see below). The third was fixed in a 4% paraformaldehyde-1% glutaraldehyde solution, embedded in an Epon-Araldite mixture, sectioned at 50 nm, stained with uranyl acetate and lead citrate, and examined by EM on a Philips 300 electron microscope.

PPF was obtained from a patient with anti-GBM nephritis (human anti-GBM PPF), who was treated by plasmapheresis, and was used by IF at a 1:3 dilution (Thorner et al. 1989b). A rabbit antiserum against human placental NC1 was purchased from Heyl Chemicals (Berlin, West Germany), and was used by IF at a 1:20 dilution. A rabbit antiserum, which had been raised by us against dog NC1 for investigation of a dog model of HN (Thorner et al. 1989b), was found to be satisfactory for use by IF at a 1:20 dilution in the present study, because of its cross-reactivity with human GBM (see Results). Indirect IF was performed using the above reagents and fluoresceinated rabbit anti-human or goat anti-rabbit IgG antiserum (Cappel, Cochraneville, PA). In some of the IF studies, tissue sections of kidney were treated for 2 h with acid urea (6 M urea, 0.1 M glycine-HCl, pH 3.5) before application of the PPF of rabbit antisera and fluoresceinated conjugates, to expose hidden NC1 determinants, as previously described (Yoshioka et al. 1985). PPF obtained from a patient who underwent plasmapheresis as treatment for familial hypercholesterolaemia, and normal rabbit serum were used as negative controls.

Kidneys were obtained from normal adult mixed-breed dogs, and patients at the time of post-mortem examination. Collagenase digests of GBM were prepared as described previously (Thorner et al. 1989b), based on modifications of earlier methods (Spiro 1967; Wieslander et al. 1983).

Gel electrophoresis was performed by SDS-PAGE (5% stacking and 8–18% linear separating gels), using the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue. Standards (Sigma) used to calculate molecular weights included phosphorylase B (92.5 K), bovine serum albumin (69 K), ovalbumin (46 K), carbonic anhydrase (30 K), soybean trypsin inhibitor (21.5 K) and lysozyme (14.3 K).

Western blotting was performed using the method of Towbin et al. 1979). Gels were first subjected to electrophoresis as above, and the separated components were then transferred to nitrocellulose at 25 volts for 18 h using a Trans-blot cell (Biorad, Richmond, California). The blots were incubated overnight with human anti-GBM PPF (1:50), rabbit anti-human NC1 antiserum (1:120) or rabbit anti-dog NC1 antiserum (1:100) and rinsed; 10^6 cpm I^{125} protein A was then added for 5 h. The blots were washed, exposed to X-ray film and developed.

A plate-binding radioimmunoassay (RIA) was employed, as described previously (Thorner et al. 1989b). Wells of microtitre plates were coated with 100 µl of human or dog NC1 (10 µg/ml), dissolved in either 0.02 M phosphate buffered saline (PBS) (145 mM NaCl, 9 mM NaH_2PO_4, 11 mM Na_2HPO_4, pH 7.2) or acid urea. The NC1 was removed, the wells were washed with PBS, and 100 µl of various dilutions of the human anti-GBM PPF or rabbit anti-NC1 antisera were added, followed by washes with PBS. Next, 15,000 cpm of I^{125} protein A (Amersham, Arlington Heights, Illinois) in 100 µl was added, the wells were washed with PBS, cut out and counted on a Beckman 300 gamma counter. The PPF and rabbit serum used as controls for the IF experiments also served as controls in the RIA studies. All wells were set up in duplicate.

Results

Some of the salient clinical features seen in both patients are shown in Table 1. On the basis of these and the findings observed on their renal biopsies (see below), a diagnosis of HN was made. How-

Table 1. Clinical features in two patients with hereditary nephritis

Patient	1	2
Sex	M	F
Age at presentation (yrs)	1.3	6
Age at time of biopsy (yrs)	2	13
Renal disease at biopsy	Haematuria, proteinuria	Haematuria
Extra-renal disease	None	Sensorineural hearing loss
Family history	Negative	Mother (proteinuria) Sister (proteinuria) Brother (sensorineural hearing loss and proteinuria)
Follow-up (yrs)	1.5	0.5
Outcome	Haematuria, increased proteinuria	Haematuria, proteinuria

Table 2. Characterization of human plasmapheresis fluid and rabbit antisera

Technique	Acid-urea	Human Anti-GBM PPF	Rabbit Anti-NC1 antisera	
			anti-human	anti-dog
IF of human GBM[1]	Absent	+	++	−
	Present	+++	+++	+++
RIA of human NC1[2]	Absent	+	+	+
	Present	++	++	++
Western blotting of human NC1	Absent	+[3]	+[4]	+[5]

[1] Intensity of staining of GBM by immunofluorescence (IF) microscopy graded as: − (negative), + (weak), ++ (moderate), +++ (strong)
[2] Radioimmunoassay (RIA) graded as: + (readings above background and falling progressively with dilution of PPF or rabbit antisera), ++ (readings greater than +)
[3] The human anti-GBM PPF bound to dimers and the 26 K and 28 K monomer components of human NC1
[4] The rabbit anti-human NC1 antiserum bound to dimers and the 24 K and 26 K monomer components of human NC1
[5] The rabbit anti-dog NC1 antiserum bound to dimers and the 26 K monomer component of human NC1

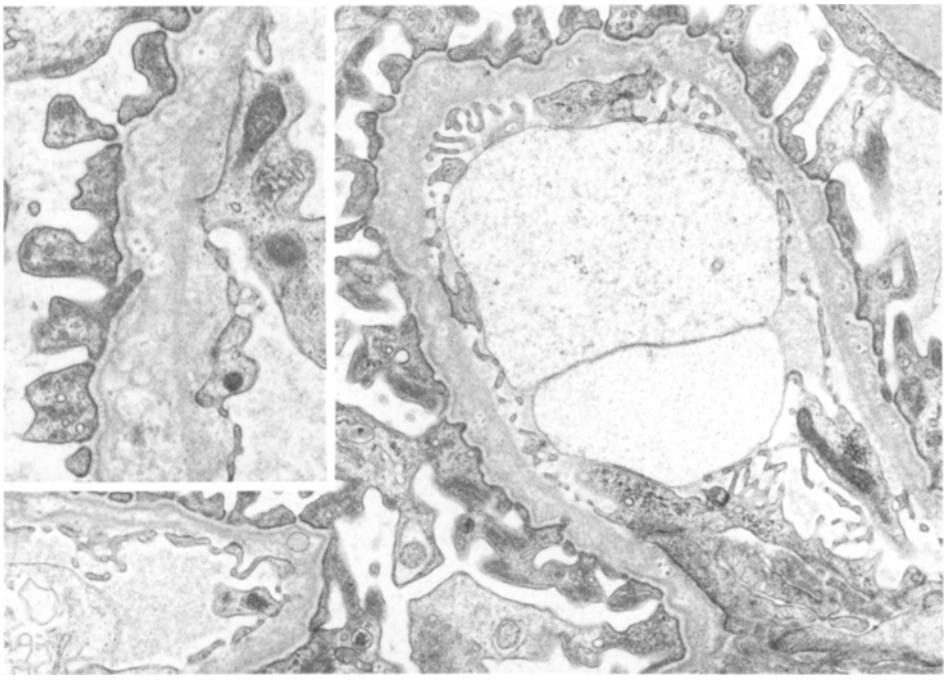

Fig. 1. Electron micrograph of glomerulus seen on renal biopsy of one of the patients reported or in the present study, showing multilaminar splitting of GBM (Mag ×15,000). The *inset* is a higher power magnification (Mag ×25,000). A similar result was obtained in the second patient

ever, using pedigree analysis, it was not possible to determine whether the HN was inherited in an X-linked or autosomal dominant fashion in either patient.

Renal biopsies performed on the 2 patients appeared similar. By LM, both showed normal glomeruli, by IF there was no deposition of immunoglobulins or complement in glomeruli, while by EM, there was multilaminar splitting of GBM, with electron-dense granulations between the split layers surrounded by electronlucent areas (Fig. 1).

Table 2 shows the results of characterisation

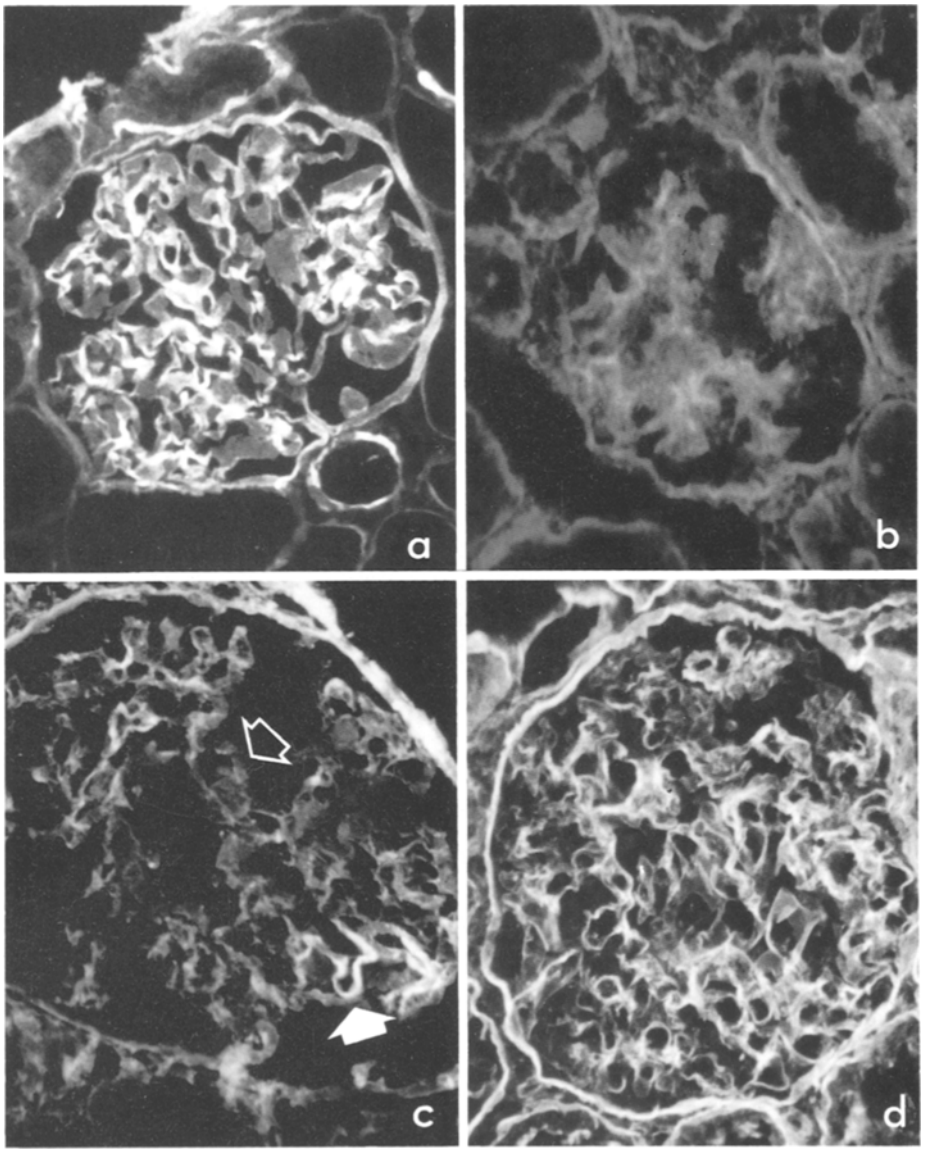

Fig. 2. Immunofluorescent photomicrographs of: **a** glomerulus of normal human kidney, treated with acid urea and stained with human anti-GBM PPF, showing global staining of GBM (Mag ×300); **b** glomerulus of the male patient with hereditary nephritis, treated with acid urea and stained with human anti-GBM PPF, showing no staining of GBM (Mag ×300); **c** glomerulus of the female patient with hereditary nephritis, treated with acid urea and stained with human anti-GBM PPF, showing segmental staining of GBM. The solid arrow points to positively staining GBM, and the *open arrow* to GBM that fail to stain (Mag ×300); **d** glomerulus of the female patient with hereditary nephritis, treated with acid urea and stained with rabbit anti-dog NC1 antiserum, showing global staining of GBM (Mag ×300). A similar result was obtained using the rabbit anti-human NC1 antiserum

of the Human anti-GBM PPF, the rabbit anti-human NCl antiserum, and the rabbit anti-dog NCl antiserum. By IF, the PPF produced weak global staining of GBM of normal human kidney before, and intense staining after, treatment of tissue sections with acid urea (Fig. 2a), indicating that it detected both exposed and hidden NC1 determinants. By RIA, the PPF reacted with human NC1, particularly when the NC1 was dissolved in acid urea (Fig. 3A). By SDS-PAGE, human NC1 was separated into 46 K and 49 K dimer, and 24 K, 26 K and 28 K monomer components (Fig. 4A), which bound by Western blotting the PPF except for the 24 K monomer (Fig. 4B). The 28K band showed the greatest degree of binding.

For the rabbit anti-human NC1 antibody, by IF, moderate global staining of GBM of normal human kidney was seen before, and intense staining after, acid urea treatment, indicating that both exposed and hidden NC1 determinants were also detected by this reagent. By RIA, this antibody reacted with human NC1, and reactivity was enhanced in the presence of acid urea (Fig. 3B). However, no reactivty was seen by RIA with other GBM constituents, including the triple helical portion of collagen type IV, laminin and fibronectin (data not shown). By Western blotting, this reagent bound strongly to dimers and to the 26 K monomer component of human NC1, and weakly to the 24 K monomer (Fig. 4C).

By IF, the rabbit anti-dog NC1 antibody produced intense global staining of GBM, but only after acid urea treatment, indicating that it detected hidden NC1 determinants. By RIA, it

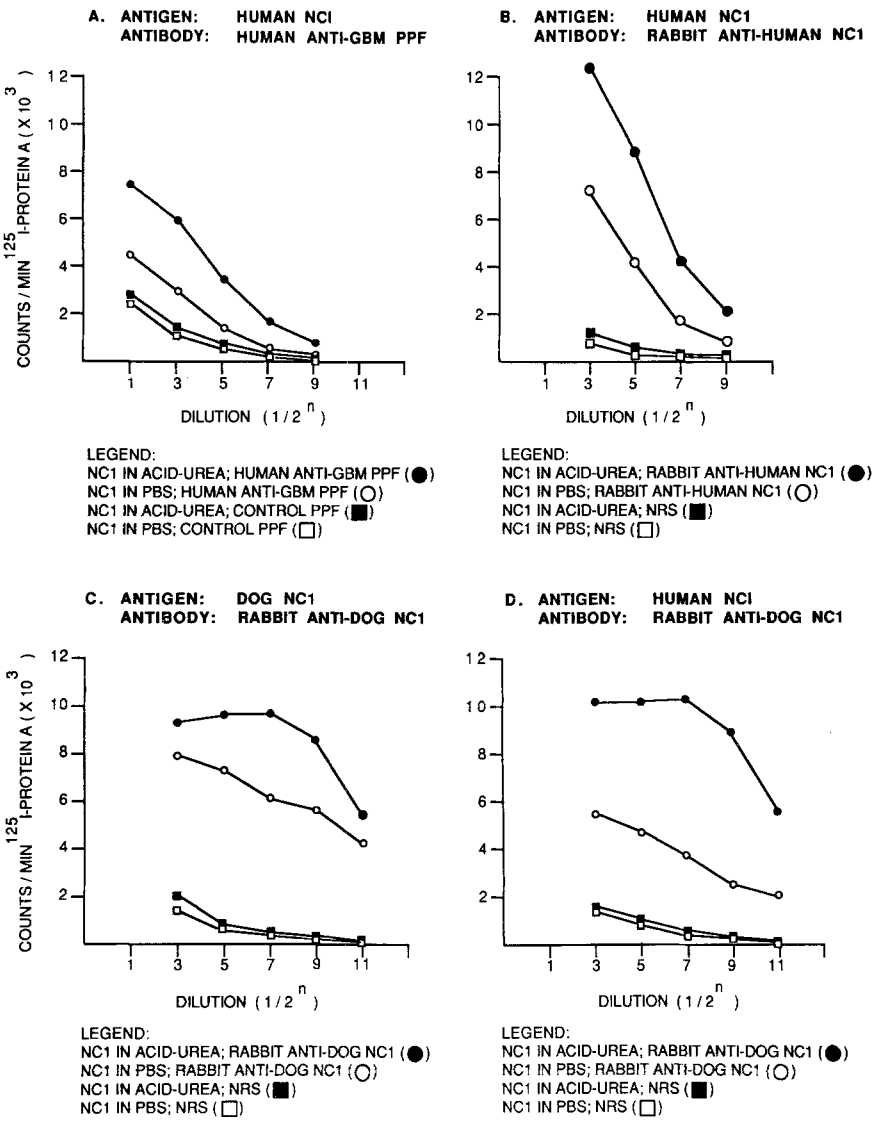

Fig. 3. Plate-binding radioimmunoassay, showing reactivity of: **A** human NC1 with human anti-GBM PPF; **B** human NC1 with rabbit anti-human NC1 antiserum, **C** dog NC1 with rabbit anti-dog NC1 antiserum; **D** human NC1 with rabbit anti-dog NC1 antiserum. Wells of microtitre plates were coated with a collagenase digest of GBM containing NC1, dissolved in either PBS or acid urea, followed by human PPF, rabbit anti-NC1 antiserum, normal rabbit serum, and then I^{125}-protein A

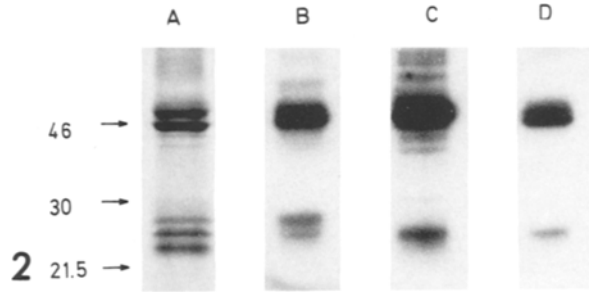

Fig. 4. Gel electrophoresis and Western blotting of NC1 in a collagenase digest of GBM of normal humans. In a first experiment, a collagenase digest of human GBM was analyzed by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue (**A**). In a second experiment, samples of the collagenase digest were first analyzed by SDS-PAGE, then transferred to nitrocellulose (Western blotting) and treated with (**B**) human anti-GBM PPF, (**C**) rabbit anti-human NC1 antiserum, or (**D**) rabbit anti-dog NC1 antiserum, followed by I^{125}-protein A. The molecular weights of standards are shown on the left

reacted equally with dog and human NC1, and reactivity was enhanced when the NC1 was dissolved in acid urea (Fig. 3C and D). As with the rabbit anti-human NC1 antiserum, no reactivity by RIA was seen with other constituents of GBM (Thorner et al. 1989b). By Western blotting, the dimers and the 26 K monomer component of human NC1 bound this antiserum (Fig. 4D).

There was no staining by IF of GBM in 6 glomeruli seen on the renal biopsy of the male patient with HN using the human anti-GBM PPF, either before or after treatment with acid urea (Fig. 2b). In the female patient, a spectrum of positivity of GBM staining was present in the 15 glomeruli seen on the renal biopsy: a few glomeruli showed staining of fewer than 25% of GBM (Fig. 2c), most showed staining of 25–75% of GBM, and a few showed staining of more than 75% of GBM. Glob-

al staining was observed on the renal biopsies of both patients using the rabbit antiserum to human NC1, both before and after acid urea treatment (Fig. 2d), and with the rabbit antiserum to dog NC1, but only after acid urea treatment.

Discussion

Collagen type IV is found exclusively in basement membranes and was initially felt to contain only α1(IV) and α2(IV) chains (Martinez-Hernandez and Amenta 1983). More recently, two novel chains have been discovered, which have been designated α3(IV) and α4(IV) (Butkowski et al. 1987; Langeveld et al. 1988; Saus et al. 1988). The α chains form a triple helix, constituting the main portion of the molecule, and a separate triple helix at the N-terminus, referred to as the 7 S domain. A collagenase-resistant region, the NC2 domain, joins the 7 S domain to the main helix, while a larger collagenase-resistant region, the NC1 domain, is located at the C-terminus (Bächinger et al. 1982; Weber et al. 1984). The NC1 domain can be isolated by collagenase digestion of GBM and exists as a hexamer under native conditions, with a molecular weight of 170 K (Weber et al. 1984; Wieslander et al. 1984b). Following SDS-PAGE, two sets of bands are seen, dimers with a molecular weight of about 50 K, and monomers, located at 24 K, 26 K and 28 K (Wieslander et al. 1985; Butkowski et al. 1985; Kleppel et al. 1986). GPA has been shown to be contained in the NC1 domain, since the latter reacts with serum obtained from patients with Goodpasture's syndrome (Wieslander et al. 1984a; Wieslander et al. 1984b; Wieslander et al. 1985). Most of these sera have been reported to bind to the 28 K component of NC1 by Western blotting (Wieslander et al. 1985; Butkowski et al. 1985; Kleppel et al. 1986; Butkowski et al. 1987) and, therefore, GPA is felt to reside in this monomer. However, some sera have shown binding to all 3 monomers (Kleppel et al. 1987). The human anti-GBM PPF used in our study possessed reactivity with the 28 K and 26 K monomers of NC1, the rabbit anti-human NC1 antiserum reacted only with the 26 K and 24 K monomers, and the rabbit anti-dog NC1 antiserum reacted only with the 26 K monomer.

In the case of the male patient with HN, GBM failed to stain using the human anti-GBM PPF. This result was similar to that reported by some workers using various sera obtained from patients with Goodpasture's syndrome by IF (Olson et al. 1989; Jenis et al. 1981; McCoy et al. 1982; Jeraj et al. 1983). It was suggested that the GPA constituent of the NC1 domain was absent from GBM of male patients with HN. In the case of the female patient, the human anti-GBM PPF produced segmental staining of GBM by IF. This result was identical to that previously obtained by us using the same reagent to stain GBM of carrier female dogs in a family with Samoyed hereditary glomerulopathy (SHG), an experimental model for X-linked HN (Thorner et al. 1989a). Segmental staining was interpreted to result from inactivation of either the normal X chromosome, resulting in negative staining of GBM, or of the mutant X chromosome, resulting in positive staining. Hence, we postulate that the segmental pattern of staining seen in GBM of the female patient in our study implied an X-linked mode of inheritance of HN.

The demonstration of segmental staining of GBM in the female patient with HN differed from results obtained by others using serum obtained from patients with Goodpasture's syndrome for staining by IF of GBM of female patients with HN (Olson et al. 1980; Jenis et al. 1981; McCoy et al. 1982; Jeraj et al. 1983). In these studies, GBM were reported not to stain in 4 of 11 (36%) females and showed positive staining in the remaining 7. The pattern was described as global in 2, but was not described in the remaining 5. There are several possible reasons why a segmental pattern has not previously been commented on. First, different sera were used for IF staining; second, staining of GBM was evaluated solely as positive or negative; and third, although young carrier female dogs with SHG showed segmental staining of GBM, we recently found that older carrier females showed global staining (unpublished observations), implying that the staining pattern may evolve from segmental to global. This observation may reflect the phenomenon of age-related reactivation of an X-linked gene, which has been recently reported (Wareham et al. 1987). Therefore, we speculate that GBM of female patients with HN may show either segmental or global staining of GBM for GPA, depending on the age of the patient when a renal biopsy is performed.

The absence of staining by IF of GBM (i.e. global or segmental) seen in our 2 patients with HN using the human anti-GBM PPF suggested that the 26 K and 28 K monomers of NC1 were either absent from GBM, or were present in a structurally altered form, leading to a diminution in their immunological reactivity. However, the positive staining of GBM seen with the rabbit anti-NC1 antisera indicated that the 26 K monomer was present. Together, these observations have led us to make two proposals: first, the 26 K monomer

of NC1 in GBM was structurally altered; and second, the 28 K monomer was either absent, or present but altered. It was not possible to obtain more information about the second of these proposals, since the rabbit antibodies used in this study did not react with the 28 K monomer of NC1. Furthermore, since none of our antibodies was specific for the 24 K monomer, we could not assess it in this study. Another study which characterized NC1 in GBM of patients with HN demonstrated that the 24 K and 26 K monomers of NC1 were present but that the 28 K monomer was absent; it was suggested that this was the underlying defect (Kleppel et al. 1987). This result was supported by the observation that serum obtained from an HN patient who developed anti-GBM antibody following renal transplantation contained antibody directed against the 28 K monomer of NC1 (Kleppel et al. 1987). However, another such serum, which did not stain GBM of patients with HN by IF, was shown to react with the 26 K monomer of NC1, implying that it was abnormal in HN (Kashtan et al. 1986). Thus, it would appear that more than one monomer of NC1 is abnormal in human HN. Others have reached a similar conclusion (Savage et al. 1989).

The results obtained in the present study have allowed us to speculate on the pathogenesis of human HN. If absence of the 28K monomer of NC1 is the primary defect in HN, one would not expect to find as well an abnormality of the 26 K monomer, as we did in our study. Furthermore, the 24 K and 26 K monomers of NC1 are derived from the α2(IV) and α1(IV) chains of collagen type IV respectively (Butkowski et al. 1987), and are coded for by separate genes on chromosome 13 (Emanuel et al. 1986; Griffin et al. 1987), whereas most cases of HN are inherited in an X-linked fashion (Hasstedt and Atkin 1983; Feingold et al. 1985). These observations suggest that the underlying defect in the X-linked form of HN may involve an enzyme, coded for by the X-chromosome, which is involved in modifying the NC1 domain, as others have suggested (Kleppel et al. 19871 Savage et al. 1989). On the other hand, absence of the 28 K monomer in HN could conceivably be secondary to abnormalities of either the 24 K and/or 26 K monomer (Kleppel et al. 1987). A primary defect of the 24 K and/or 26 K monomer of NC1 could be consistent with an autosomal form of HN.

Our studies on monomers of the NC1 domain in GBM have been carried out on only 2 patients with HN, in whom pedigree analysis failed to determine the mode of inheritance. Further work should include performance of IF studies on GBM of additional patients with HN, as well as biochemical studies of the NC1 domain in their GBM.

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Recently, we have come across 2 papers which describe segmental staining of GBM in female patients with hereditary nephritis (Gubler et al. Progress in basement membrane research. Renal and Related Aspects in Health and Disease: M.C. Gubler, M. Sternberg (eds) 1988, John Libbey Eurotext Ltd. pp 177–182, and Kleppel et al., Lab Invest 61:278–289, 1989).