Virchows Arch A (1987) 411:79–85

Virchows Archiv A

Quantitation of anionic sites in glomerular capillar basement membranes of Samoyed dogs with hereditary glomerulopathy

Paul S. Thorner[1,2], Barbara Jansen[3]**, Jon Liang[1]*, Reuben Baumal[1,2]*, and Victor Valli[3]***

[1] Departments of Pathology, The Hospital for Sick Children 555 University Avenue Toronto, Ontario M5G1X8, Canada
[2] University of Toronto, Toronto,
[3] Ontario Veterinary College, Guelph, Canada

Summary. Samoyed hereditary glomerulopathy (SHG) is an X-linked dominant disease characterized by proteinuria and renal failure in affected male dogs. Electron microscopic examination of glomerular capillary basement membranes (GCBM) shows widespread multilaminar splitting of the lamina densa, identical to that in Alport's syndrome. Anionic sites in GCBM of three affected males and five unaffected dogs were labeled using polyethyleneimine to determine whether proteinuria was associated with an alteration in their number. No significant differences were noted in the number of anionic sites in the lamina rara externa, whereas small but statistically significant increases were seen in the number of sites in the lamina rara interna of affected males. In the lamina densa, affected males showed a striking increase in anionic sites, particularly in regions of GCBM which were split. Thus, although proteinuria in some glomerular diseases has been attributed to a reduction in anionic sites in GCBM, this was not so in SHG.

Key words: Anionic sites – Heparan sulfate proteoglycans

Introduction

Glomerular capillary walls consist of epithelial and endothelial cells and glomerular capillary basement membranes (GCBM), and act as a filtration barrier to constituents of the blood (Farquhar 1975; Kanwar 1984). Molecules are prevented from passing through glomerular capillary walls from blood into urine, with the result that urine is virtually protein free (Caulfield and Farquhar 1974; Brenner et al. 1978; Rennke et al. 1978). The passage of anionic molecules is impeded more than that of neutral or cationic molecules, suggesting the existence of a charge-selective barrier. One component of this barrier consists of anionic sites in GCBM which are composed largely of heparan sulfate proteoglycan (HSPG). These sites can be detected using two approaches: 1) histologically by enzyme digestion studies (Kanwar and Farquhar 1979a, b; Vernier et al. 1983), immunologic probes (Hassell et al. 1980; Klein et al. 1983; Mynderse et al. 1983), or cationic dyes (Caulfield and Farquhar 1974, 1978; Schurer et al. 1977, 1978; Caulfield 1979; Kanwar and Farquhar 1979a; Reale et al. 1983; Pilia et al. 1985) and 2) by biochemical analysis (Kanwar and Farquhar 1979c; Parthasarathy and Spiro 1981).

It has been suggested that proteinuria of glomerular origin is caused by a decrease in the HSPG content of GCBM. Such a decrease has been documented by some (Bohrer et al. 1977; Caulfield and Farquhar 1978; Bridges et al. 1982; Mahan et al. 1983; Mynderse et al. 1983) but not all workers (Caulfield 1979; Kanwar et al. 1981; Kanwar and Jakubowski 1984) in minimal lesion disease (MLD) and its experimental counterpart induced in rats by the administration of puromycin aminonucleoside. A decrease in HSPG anionic sites in GCBM has also been demonstrated in diabetes mellitus, both in man (Carrie and Myers 1980; Parthasarathy and Spiro 1982) and in experimental animals (Rohrbach et al. 1982), and in patients with congenital nephrotic syndrome (Vernier et al. 1983).

We have been studying a spontaneously occur-

* This study was supported by a grant from the Medical Research Council of Canada
** P.S. Thorner and B. Jansen were recipients of MRC fellowships

Offprint requests to: P. Thorner at the above address

ring hereditary glomerulopathy in Samoyed dogs (SHG) which is characterized in affected males by proteinuria beginning at about 3 months of age, renal failure, and death at 12 to 15 months (Bernard and Valli 1977; Jansen et al. 1984). The disease is transmitted as an X-linked dominant trait via carrier females that show variable degrees of proteinuria and do not develop renal failure (Jansen et al. 1986). The morphologic changes in GCBM seen by electron microscopy (EM) in affected male dogs are identical to those in Alport's syndrome, namely, extensive splitting of the lamina densa (LD) of GCBM into multiple layers, often with electron-dense particles between the layers (Jansen et al. 1984). In contrast, carrier females show a milder picture, with only restricted areas of splitting of GCBM. In the present study, we investigated whether an altered number of anionic sites of GCBM was associated with proteinuria in male dogs affected with SHG.

Materials and methods

Eight Samoyed dogs from 2 l in which the mothers were known carriers of SHG were killed at 4 months of age. Before sacrifice, the presence of protein in the urine was assessed using commercial dipstick reagent strips (Ames, Rexdale, Ontario, Canada) and 24 h collections were quantitated turbidimetrically using 20% sulfosalicylic acid. Samples of kidney were fixed in 4% paraformaldehyde-1% glutaraldehyde, cut at 50 nm thickness for routine EM, stained with uranyl acetate and lead citrate, and examined at 60 kV using a Philips 300 electron microscope. For labeling of anionic sites in GCBM, portions of kidney were fixed in 1% paraformaldehyde for 1 h at 4° C and then transferred to 0.1 M phosphate buffer. The tissue was then embedded in polyethylene glycol (MW 1000) at 37° C in order to avoid distortion which was observed in frozen sections and 10 μ thick sections were cut at room temperature using a rotary microtome. Staining with polyethyleneimine (PEI) (MW 1200) (Polysciences, Warrington, PA, USA) was performed using a modification of the method of Schurer et al. (1978). The sections was suspended overnight in 1% PEI, incubated in 2% phosphotungstic acid-0.1% glutaraldehyde for 2 h, post-fixed in 4% paraformaldehyde-1% glutaraldehyde followed by 1% osmium tetroxide, and flat-embedded in Epon-Araldite using pre-hardened capsules. Blocks were cut at 1 μ until at least one glomerulus was recovered in a section, at which point they were cut at 50 nm thickness, picked up on copper grids, and examined unstained at 60 kV using a Philips 300 electron microscope. Sections were examined unstained, as post-staining with uranyl acetate and lead citrate tended to obscure anionic sites, making quantitation difficult.

Photographs were taken at random of at least 11 capillary loops from 2 or 3 glomeruli of each dog. The negatives were mounted on a mechanical stage linked to a camera and connected to an IBAS-2 computer. The number of anionic sites was quantitated manually in each of the LD, lamina rara interna (LRI), and lamina rara externa (LRE) of GCBM. The number of counts was determined concurrently for LD, LRI, and LRE while scanning a given portion of GCBM, by storing them in separate channels for each of these layers. The film negative was advanced using the mechanical stage to bring a fresh segment of GCBM into view. Indistinct sites were not quantitated. LRI and LRE were considered to be the electron lucent regions between endothelial and epithelial cells respectively and the first electron-dense band in the GCBM. The remainder of the GCBM was considered to be LD. The anionic sites in split regions of GCBM of dogs with SHG were quantitated separately from those in morphologically normal regions. Anionic sites were quantitated in whole capillary loops or parts of loops for a length of at least 15.2 µm of GCBM and an area of at least 2 µm^2 of GCBM. The IBAS-2 was also used to determine lengths and areas of GCBM examined. Comparison of the mean number of anionic sites in the LRE, LRI, and LD of GCBM of three affected males and five unaffected dogs was performed using Student's t test.

Results

Proteinuria was seen in 3 of the 8 dogs, 3 males having random 24 h urinary protein excretions of 31.8 mg/kg, 105.9 mg/kg and 184.5 mg/kg body weight (normal <22 mg/kg). In these males, EM demonstrated widespread splitting of the LD of GCBM into multiple layers, with electron-dense particles between the layers (Fig. 1), so that they were considered to be affected by SHG (Jansen et al. 1984). The remaining 5 dogs (1 male, 4 females) were unaffected because they showed no proteinuria (24 h urinary protein excretion $=0$ mg) and EM failed to demonstrate any abnormality in their GCBM.

Labeling of glomeruli of affected males and unaffected dogs with PEI and examination by EM revealed punctate staining over GCBM and mesangial regions. In addition, cell surfaces of podocytes and endothelial cells were labeled (Fig. 2). In unaffected dogs, staining was most marked in the LRE of GCBM, but was also seen in the LRI. Only sporadic staining was seen in the LD. Both the LRI and LD staining was variable from capillary loop to loop, whereas the LRE staining was much more consistent, as has been noted by others (Vernier et al. 1983; Pilia et al. 1985). Male dogs affected with SHG showed staining of anionic sites in both LRE and LRI, but there was also staining in the LD, particularly in regions of splitting of GCBM (Fig. 3).

The results of quantitation of anionic sites in given lengths of LRE, LRI, and LD of GCBM are shown in Table 1. No significant differences were seen in the number of anionic sites in the LRE of GCBM of the three affected males compared to the five unaffected dogs ($P>0.05$). A small but significant increase was noted in the number of anionic sites in the LRI of non-split and split regions of GCBM of the affected male dogs compared to the unaffected dogs ($P<0.05$). The LD of GCBM of affected males showed an

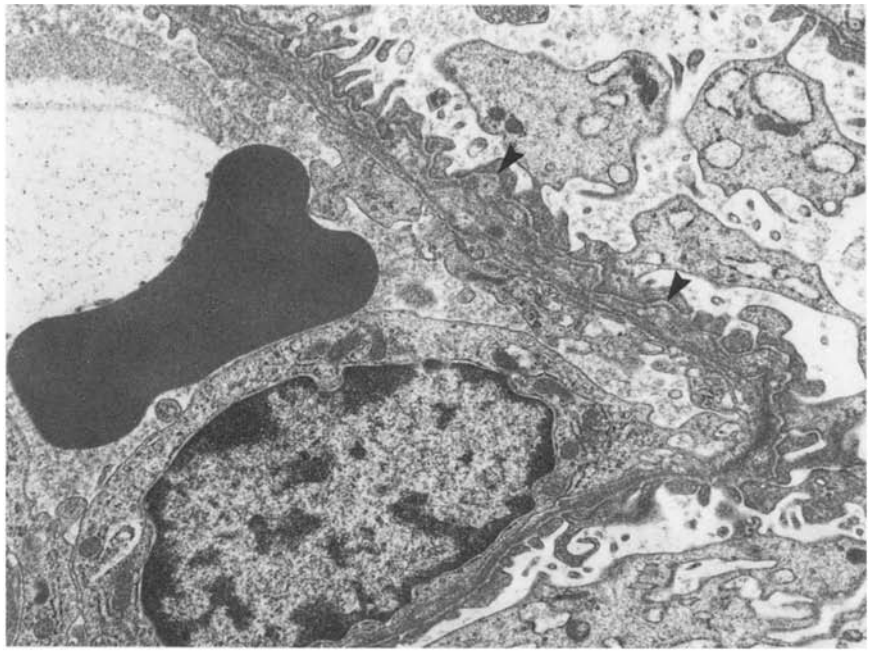

Fig. 1. Electron micrograph of glomerulus from a dog affected with Samoyed hereditary glomerulopathy. The glomerular capillary basement membrane shows splitting of the lamina densa (*arrows*) identical to that in Alport's syndrome. Stained with uranyl acetate and lead citrate. (×10500)

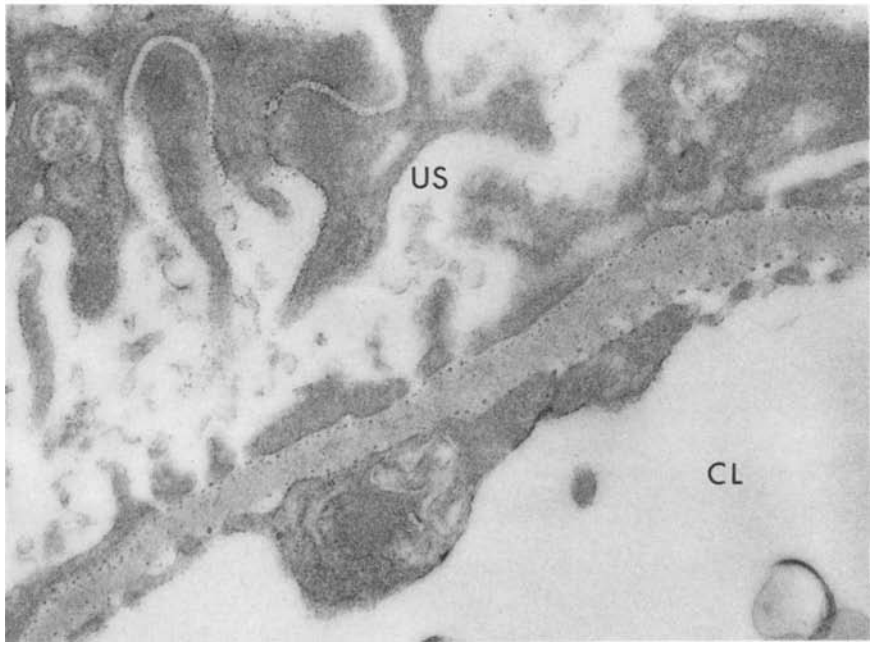

Fig. 2. Electron micrograph of glomerulus from a male dog not affected with Samoyed hereditary glomerulopathy, in which anionic sites in the glomerular capillary basement membrane have been labeled with polyethyleneimine. There is regular staining of the lamina rara externa and less regular staining of the lamina rara interna and lamina densa. Staining along cell surfaces is also apparent. (CL-capillary lumen; US-urinary space) (×32000)

increase in the number of anionic sites compared to the unaffected dogs, with a greater increase seen in regions of GCBM that demonstrated splitting ($P<0.01$). The increased standard deviation seen in the LD in split regions of GCBM reflected different degrees of splitting in the three affected males.

In the three affected males, the degree of splitting of the LD (38%, 57%, and 71% of the length of glomerular capillary loops examined) correlated with the amount of proteinuria (31.8 mg/kg/24 h, 105.9 mg/kg/24 h, and 184.5 mg/kg/24 h respectively) and with the number of anionic sites in split regions of GCBM. No correlations were noted between the amount of proteinuria and the number of anionic sites in the LRE or LRI of split regions of GCBM or the LRE, LRI, or LD of non-split regions of GCBM.

GCBM of affected male dogs were thickened in regions which showed splitting. The marked increase in the number of anionic sites in the LD in these regions could have been due solely to their

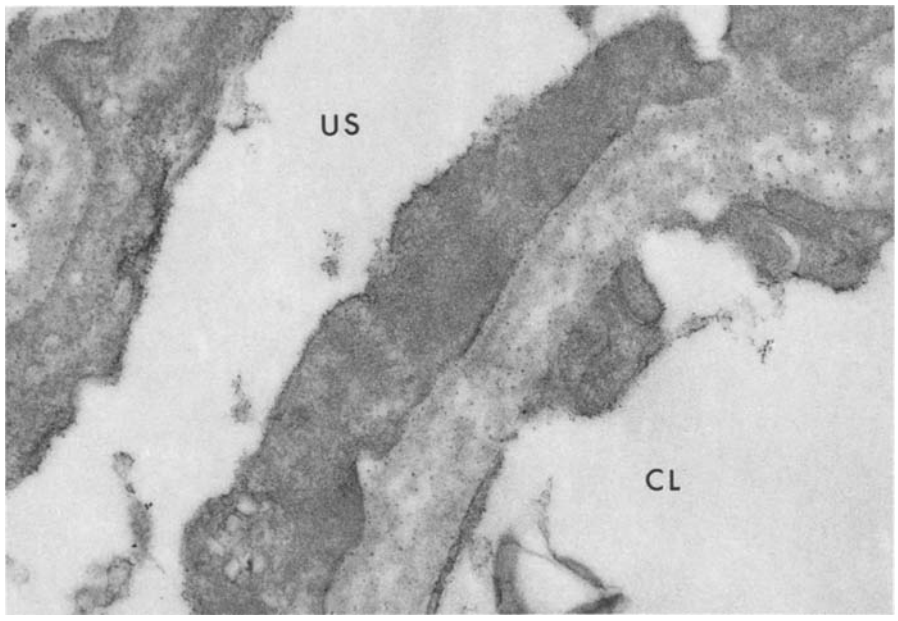

Fig. 3. Electron micrograph of glomerulus from a male dog affected with Samoyed hereditary glomerulopathy, in which anionic sites in the glomerular capillary basement membrane have been labeled with polyethyleneimine. A staining pattern similar to that seen in Fig. 2 is present in the lamina rara externa and interna. However, there is a marked increase in the number of anionic sites in the lamina densa. (CL-capillary lumen; US-urinary space) (×30000)

Table 1. Results of quantitation of anionic sites in glomerular capillary basement membranes

Status (sex) of dogs	No of glomerular capillary loops examined	Length (μm) of glomerular capillary basement membrane measured	No of anionic sites (mean ± SD)			
			per μm basement membrane in			per μm² basement membrane in LD
			LRE	LRI	LD	
Unaffected						
1 (F)	15	48.7	31.5±3.1	17.1±3.1	6.8±2.3	ND
2 (F)	9	15.2	27.8±3.2	17.4±2.9	5.4±0.8	ND
3 (F)	22	58.6	30.3±4.8	17.9±2.9	6.1±1.7	ND
4 (F)	19	38.7	28.1±4.6	16.7±2.1	4.6±1.1	ND
5 (M)	15	54.3	28.9±5.8	17.7±3.0	5.4±1.4	ND
Average	–	–	29.3±3.4	17.4±2.5	5.7±1.4	ND
Affected						
1 (M)	26	50.9 (non-split)	29.8±3.4	22.8±4.2	12.4±3.6	174.8±54.6
2 (M)	23	31.2 (non-split)	29.8±4.2	22.6±3.2	11.8±4.0	186.6±40.7
3 (M)	17	16.6 (non-split)	32.5±2.9	24.5±3.2	12.4±4.0	193.0±39.3
Average	–	–	30.7±2.5	23.3±2.6	12.2±2.4	184.8±29.8
1 (M)	26	30.7 (split)	29.4±4.3	22.5±3.8	83.7±32.4	446.0±66.2
2 (M)	23	41.8 (split)	31.5±3.6	21.7±3.2	100.0±32.0	505.8± 8.3
3 (M)	17	40.4 (split)	32.2±4.9	23.7±3.7	128.2±54.1	722.4±80.8
Average	–	–	31.0±3.1	22.6±2.7	104.0±30.1	558.1±53.6

Abbreviations: LRE = lamina rara externa; LRI = lamina rara interna; LD = lamina densa

increased width. To determine whether this was the case, the number of anionic sites in the LD was quantitated per unit area in split and non-split portions of GCBM of affected male dogs. Had increased width alone been responsible for the increased anionic site content of GCBM, the number of sites per unit area of GCBM would have been the same in split and non-split regions of GCBM, but this was not the case. Split portions of GCBM still showed an increased number of anionic sites in the LD compared to non-split regions ($P < 0.01$), reflecting an absolute increase in their number.

Discussion

Various studies have demonstrated that GCBM function as both a size- and charge-selective barrier (Caulfield and Farquhar 1974; Brenner et al. 1978;

Rennke et al. 1978). The charge barrier is anionic, with the result that there is selective restriction in the passage of negatively charged molecules, including plasma proteins, into urine. Anionic sites have been demonstrated by EM as granules, 10–20 nm in diameter, in the mesangium and GCBM, mainly in the LRE, but also in the LRI and rarely in the LD, using electron-dense cationic probes such as ruthenium red, Alcian blue, PEI, lysozyme, cationic ferritin, and safranine O (Caulfield 1979; Farquhar 1978; Schurer et al. 1978; Kanwar and Farquhar 1979a, b; Reale et al. 1983; Pilia et al. 1985). In the LRE, the granules are distributed regularly at intervals ranging from 42 nm to 100 nm (Caulfield and Farquhar 1978; Schurer et al. 1978; Caulfield 1979; Kanwar and Farquhar 1979a, b; Reale et al. 1983; Vernier et al. 1983; Pilia et al. 1985). Some workers have shown that filaments connect the granules in the LRE to the podocyte cells surface and have postulated that anionic sites play a role in cell attachment (Caulfield 1979; Kanwar and Farquhar 1979b). A similar role has been suggested for anionic sites which have been demonstrated in other non-filtering basement membranes, including renal tubules, capillaries of the lung, and the dermal-epidermal junction of skin (Schurer et al. 1978; Caulfield 1979; Charonis and Wissig 1983; Ferrara et al. 1985; Manabe and Ogawa 1985). An anti-clogging function has also been proposed for anionic sites, since their neutralization leads to decreased permeability of GCBM and accumulation of tracers in them (Kanwar and Rosenzwieg 1982).

Anionic sites in GCBM have been shown to contain heparan sulfate proteoglycan by both enzymatic digestion studies (Kanwar and Farquhar 1979a, b; Vernier et al. 1983) and immunohistochemistry (Hassell et al. 1980; Klein et al. 1983; Mynderse et al. 1983). The 10 to 20 nm diameter granules seen in GCBM by cationic dye labeling are considered too small to represent the entire HSPG molecule in its in vivo state (Hascall 1980; Reale et al. 1983). Moreover, distances of 42–100 nm between anionic sites are too wide to prevent molecules, such as albumin, from passing through GCBM. Hence, it was postulated that the polycationic dyes and/or dehydration agents used for labeling of GCBM caused the HSPG molecules to collapse, thereby producing the granular staining seen by EM (Reale et al. 1983). Monocationic reagents, such as safranine O or modified Alcian blue, produce a somewhat different appearance, namely multi-branching filamentous structures separated by about 10 nm (Reale et al. 1983). This may be a more accurate representation of the in vivo state of HSPG. Although the punctate staining of GCBM seen following treatment with PEI may be an artefact, it is reproducible and lends itself to quantitation, as done by Vernier et al. (1983), using human kidneys ranging from fetal to adult. These workers enumerated 23.8 ± 6.8 anionic sites/μm GCBM in the LRE. Sites in the LRI and LD were not tabulated. A similar result was obtained using lung alveolar basement membranes (Ferrara et al. 1985). Pilia et al. (1985) quantitated anionic sites in the LRE and LRI of GCBM of normal rats and found that the results obtained varied with the technique used (i.e., perfusion or immersion). With perfusion, they observed 21 sites/μm GCBM in the LRE, but this figure dropped to 11 sites/μm GCBM with immersion. In addition, they found 20 sites/μm GCBM in the LRI using perfusion, but only 10 sites/μm GCBM using immersion. In comparison, our results using immersion with normal dog GCBM demonstrated 29.3 ± 3.4 sites/μm GCBM in the LRE and lower values, namely 17.4 ± 2.5 and 5.6 ± 1.4 sites/μm GCBM, in the LRI and LD respectively. These results were consistent with previous qualitative observations which showed that most labeling of anionic sites using cationic dyes occurred in the LRE (Caulfield and Farquhar 1978; Schurer et al. 1978; Caulfield 1979; Kanwar and Farquhar 1979a, b; Reale et al. 1983; Vernier et al. 1983; Pilia et al. 1985).

The possible role of an altered HSPG content of GCBM in producing proteinuria of glomerular origin has been explored in various systems. Studies of MLD in man have shown increased permeability to negatively charged molecules such as albumin, which is consistent with a diminution in the number of anionic charge sites of GCBM (Carrie and Myers 1980; Bridges et al. 1982). Studies performed in the experimental counterpart of MLD, namely rats treated with puromycin aminonucleoside, have shown increased clearance of anionic dextrans (Bohrer et al. 1977). In addition, studies on human diabetes mellitus have shown increased clearance of negatively charged molecules such as albumin and a decrease in clearance of neutral dextrans, which is consistent with a loss of anionic sites from GCBM (Carrie and Myers 1980). This has been confirmed in biochemical studies which have shown a decreased HSPG content of human GCBM in diabetes (Parthasarathy and Spiro 1982). In experimental studies, the basement membrane producing EHS tumor showed a 50–80% decrease in the HSPG content of extracellular matrix when transplanted into diabetic mice (Rohrbach et al. 1982). Hence, it has been sug-

gested that the proteinuria of diabetes is related to the lowered HSPG content of GCBM (Parthasarathy and Spiro 1982; Rohrbach et al. 1982). Finally, decreased numbers of anionic sites have been demonstrated in the LRE of GCBM in congenital nephrotic syndrome (Vernier et al. 1983) and this was postulated to reflect a failure in their development.

We used PEI to label anionic sites of GCBM for a number of reasons (Schurer et al. 1977). First, there was little non-specific binding of PEI to GCBM, as seen with cationic ferritin. Second, PEI could be used by immersion staining of fixed tissue (Vernier et al. 1983), in contrast to agents such as ruthenium red and Alcian blue, which yielded satisfactory results only by staining of fresh tissue, preferably using perfusion techniques. In the present study, we found that satisfactory labeling with PEI could be obtained after light fixation of tissue, while higher concentrations of fixative or longer times of fixation were detrimental to preservation of anionic sites. We did not compare different times of incubation in PEI or different molecular weights of PEI even though these variables have been shown to influence absolute site counts (Vernier et al. 1983; Pilia et al. 1985) because we were interested only in studying the relative difference, if any, in the number of anionic sites of GCBM of unaffected and affected dogs under identical conditions.

The proteinuria we observed in male dogs affected with SHG was not associated with a decrease in anionic sites in GCBM, as has been noted in other renal diseases (Bohrer et al. 1977; Caulfield and Farquhar 1978; Carrie and Myers 1980; Bridges et al. 1982; Parthasarathy and Spiro 1982; Rohrbach et al. 1982; Mahan et al. 1983; Mynderse et al. 1983; Vernier et al. 1983). No information is available on anionic sites in GCBM in the case of human hereditary nephritis. In our study, affected males showed an increase in the number of anionic sites in the LD. Detection of increased numbers of anionic sites might have several explanations. First, in some proteinuric states, such as passive Heymann nephritis, increased numbers of anionic sites have been observed between podocyte slit pores and immune complexes (Suzuki et al. 1983). This finding was interpreted as reflecting regeneration of anionic sites in the GCBM in response to immunologic injury. Injury to blood vessels (i.e., experimental de-endothelialization) also produced an increased number of anionic sites during repair (Wight and Ross 1975). In nephrotoxic serum nephritis, anionic sites were demonstrable in the split regions of LD, about 24 h after injury (Pilia et al. 1983). Their appearance coincided with a drop in proteinuria, suggesting a repair phenomenon. Hence, the increased number of anionic sites in affected males with SHG might reflect a response of GCBM to injury. Second, previously hidden anionic sites might have been exposed in GCBM in SHG. In various renal diseases in man, anionic sites in the LD have been found to increase with the level of proteinuria (Hirsch et al. 1981), and this may reflect the unmasking of such anionic sites. Along a similar line of reasoning, the multilaminar splitting of GCBM in SHG may permit greater penetration of PEI, resulting in the staining of anionic sites which are hidden in normal GCBM. Third, negatively charged serum proteins may have become trapped in abnormal GCBM of SHG (Hirsch et al. 1981). Enzyme digestion studies (Kanwar and Farquhar 1979a, b; Vernier et al. 1983) would be helpful to determine which (if any) proteoglycan was contained in these anionic sites.

In conclusion, the proteinuria of SHG cannot be attributed to a decrease in the number of anionic sites in GCBM. In addition, these studies do not address the issue of any abnormality in the structure or function of the HSPG molecule; nor do they rule out a change in the charge density of the HSPG molecule. Other less cationic probes would be needed to approach this latter issue. If the change in the number of anionic sites is a secondary phenomenon, the primary defect may lie in the abnormal synthesis or processing of some non-HSPG basement membrane component, producing both proteinuria and the abnormal ultrastructural appearance. For example, the multilaminar splitting of GCBM and not the anionic site change may well be the major source of the proteinuria observed in SHG, since in this study the greater the degree of splitting of GCBM, the greater the degree of proteinuria. Moreover, we recently detected an abnormality in the Goodpasture antigen (NC1 region of collagen type IV) content of GCBM of affected male dogs (unpublished observations), suggesting that the alteration in anionic site number may indeed by secondary.

Acknowledgments. This paper was prepared with the assistance of the Medical Publications Department, The Hospital for Sick Children.

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Accepted December 19, 1986