Alterations in Glomerular Anionic Sites in Autologous Phase of Canine Anti-Glomerular Basement Membrane Nephritis

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ABSTRACT

There have been a few studies on canine nephrotoxic glomerulonephritis produced by anti-glomerular basement membrane serum (AGBM). These reports have not focused on an alteration in the charge properties of glomerular basement membrane (GBM) in this disease. In this study, AGBM-treated dogs demonstrated severe or mild proteinuria and a reduction of glomerular anionic sites (AS) in the autologous phase.

Rabbit AGBM or normal rabbit serum (NRS) were given intravenously (2 ml/kg, B.W.) to 16 male beagle dogs. Light and electron microscopy and immunofluorescent test were performed on the kidneys at weeks 1, 2, 4, and 8 postinjection. An alteration of AS of GBM in peripheral, proximal, and paramesangial portions was studied quantitatively using polyethyleneimine (PEI, M.W. = 1,800) as a cationic probe by electron microscopy. Urinalysis was performed up to week 8 postinjection.

Severe or mild proteinuria developed as early as day 1 and continued until week 2. Thereafter, the degree of proteinuria decreased gradually. On weeks 4 and 8, there was no significant difference in the
proteinuria between AGBM-treated group and NRS-treated group. On weeks 1 and 2 after AGBM injection, the number of PEI granules per 1000 nm length of the lamina rara externa (LRE) of GBM in all portions was less than that in NRS-treated dogs (10.5 ± 1.8 versus 14.2 ± 2.4 in peripheral portion, 10.8 ± 1.9 versus 15.0 ± 1.4 in proximal portion, 8.4 ± 1.8 versus 13.4 ± 2.1 in paramesangial portion, on week 1, P < 0.001). On week 4, however, there was difference in the number of granules in peripheral portion of GBM. On week 8, there was no difference in the number of granules in all portions of GBM between AGBM-treated and NRS-treated dogs. Immunohistological and light and electron microscopical lesions remained in renal glomeruli of proteinuric or nonproteinuric AGBM-treated dogs.

The fact that a reduction glomerular AS occured in AGBM-treated dogs with severe or mild proteinuria and the recovery of AS in the GBM coincided with an improvement of proteinuria suggested that alterations in glomerular AS might play an important role in the pathogenesis of proteinuria in the autologous phase of canine anti-GBM nephritis.
INTRODUCTION

The immunological consequences of administration of heterologous anti-kidney serum have been described in various animals (4, 5, 6, 7, 10, 12, 16, 17, 23, 25, 28, 33). Histopathological studies have been made on the nephrotoxic nephritis in dogs by the injection of serum prepared against whole kidney or placental suspensions (2, 21). In such studies, nephrotoxic sera may contain antibodies directed not only against glomerular basement membrane (GBM) but also against other renal tissue antigens. Recent studies have indicated that anionic charge in the GBM was important for selective ultrafiltration in the renal glomeruli (9, 29, 35, 36, 42). To date, there have been a few studies on canine nephrotoxic glomerulonephritis produced by administration of anti-GBM serum (AGBM) (31, 44). These studies dealt with morphologic changes and the charge properties of GBM have not been studied in this model.

On the other hand, in the rat anti-GBM nephritis, a loss of glomerular anionic sites (AS) has been seen before detectable proteinuria (25) in the early heterologous phase. And loss of glomerular charge have been suggested to be significant in the
pathogenesis of proteinuria. But, in that report, changes of glomerular AS and urinary protein after heterologous phase have not been examined. In the canine anti-GBM nephritis, the autologous phase of this disease has been characterised by the deposition of host IgG on 7 days postinjection (44).

In the present study, autologous phase of glomerulonephritis with onset of severe proteinuria was induced by administration of AGBM in dogs and the glomerular AS were examined by electron microscopy to see the relationship between proteinuria and alteration of AS in this model.
METHODS

PREPARATION OF GBM ANTIGEN

GBM were prepared from the perfused kidneys of young beagle dogs by the method of Krisko et al. (18) as modified by Shirota et al. (31). The kidneys were decapsulated and the cortices were finely minced. The minced tissue was forced to pass through 600-, 250-, 177- and 125- μm pore-sized stainless-steel sieves with a large amount of cold physiological saline (PS). The material retained on the 125- μm pore-sized sieve was sonicated for 9 minutes and then it was allowed to pass through a 74-μm pore-sized mesh. The sample retained on this sieve was rich in glomeruli and sonicated for 30 minutes to disrupt the GBM. The resultant suspension was then lyophilised and stored at -25 °C until used.

PREPARATION OF ANTI-GBM SERUM (AGBM)

Male albino rabbits weighing about 3 kg were injected into the footpads with 60 mg of lyophilised GBM suspended in PS and emulsified in complete
Freund's adjuvant. One month later, a booster injection of the same amount of antigen was given subcutaneously without adjuvant and the animals were bled 14 days later. The sera were then pooled, decomplemented, and absorbed twice with freshly harvested dog red cells.

The specificity of AGBM was tested by indirect immunoperoxidase method with DAB as the chromogen. Briefly, paraffin sections of normal dog kidney were exposed to AGBM for 30 minutes at room temperature in a moist chamber, and after washing in phosphate-buffered saline (PBS), sections were further incubated with sheep anti-rabbit IgG antibody conjugated with peroxidase (Bethyl Laboratories, Inc., U.S.A.).

INDUCTION OF EXPERIMENTAL NEPHRITIS

Sixteen young male dogs aged 12 weeks, weighing approximately 5 kg were used. Two groups of eight animals were given AGBM or normal rabbit serum (NRS), intravenously at a dosage of 2 ml/kg body weight and their kidneys were examined by biopsy or autopsy at intervals from 1 to 8 weeks after administration of serum.
The schedule for urialysis and pathological examinations (light microscopy, immunofluorescent test and ultrastructural study for anionic sites and morphological changes) were shown in table 1.

URALYSIS

The protein content of 15-hour urine collections was measured using Ponceau S dye method (24). The protein content in each group was estimated by Wilcoxon rank sum test for non-paired observations. Any cited difference is significant at the 5% level or less.

TISSUE PREPARATIONS

For light microscopy, kidneys were fixed in 10% neutral buffered formalin, embedded in paraffin, and 4μm-sections were cut and stained with hematoxylin and eosin (H.E.). Heparan sulfate proteoglycan (HSPG) was stained by immunostaining using peroxidase-antiperoxidase (PAP) method with DAB as the chromogen. Paraffin sections were incubated with the mouse anti-HSPG monoclonal antibody (Chemicon International Inc. CA.) at room temperature for 1h.
For immunofluorescence microscopy, small samples of kidneys were frozen in hexane-dry ice acetone. Cryostat sections were made and incubated for one hour in a moist chamber with fluorescein-isothiocyanate (FITC)-conjugated antibodies, goat anti-rabbit IgG (Organon Teknika N. V.-Cappel products, West Chester, PA), sheep anti-dog IgG or sheep anti-dog complement (C3) sera (The Binding Site Ltd., Birmingham, England). After a washing in PBS for 20 minutes, sections were examined using a fluorescence microscope (Nikon Ltd, Japan). For electron microscopy, small pieces of renal cortical tissue were fixed in 2.5 % glutaraldehyde in 0.1M phosphate buffer, pH 7.4, post-fixed in 2 % osmium tetroxide in the same buffer for 2h and then dehydrated with a graded ethanol series and embedded in Quetol-812 (Nisshin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate.

ULTRASTRUCTURAL STUDY FOR ANIONIC SITES

Staining with polyethyleneimine (PEI, MW 1,800, Polysciences, Warrington, PA, USA) was performed by a minor modification of the method of Schurer et al.
(30). Freshly sampled renal cortex was cut into the 0.5 mm³ and immersed in 0.5 % PEI in PS for 30 minutes at room temperature. After washing in 0.2 M cacodylate buffer (CB), pH 7.4, 400 mOsm the blocks were reimmersed in the solution containing 2 % phosphotungstic acid (PTA) and 0.1 % glutaraldehyde (GA), pH 7.4, 400 mOsm at 4°C for one hour to obtain insoluble precipitates of PEI bound to AS. After washing three times for 10 minutes in the same CB, the blocks were post-fixed in 2 % osmium tetraoxide in 0.2 M CB at 4°C for 2 hours. Then, the blocks were dehydrated in graded ethanol and embedded in Quetol-812 (Nisshin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and examined using Hitachi-7000 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan). The PEI solution was adjusted to pH 7.4 with HCl and to 400 mOsm with sucrose. The mixture of 2 % PTA and 0.1 % GA was prepared within 2 hours before use. The osmolality of this solution was adjusted to 400 mOsm with sucrose and its pH was adjusted to 7.4 with concentrated KOH solution.

MORPHOMETRIC ANALYSIS OF AS
For the quantitative analysis of AS in different portions of GBM, the AS were counted within the lamina rara externa (LRE) and lamina rara interna (LRI) of the capillary walls and also in the LRE of the paramesangial region; A total of 30 ~ 40 portions in 3 ~ 4 glomeruli were observed on each dog. The specimens were photographed at a magnification of x 7,000 and then printed at a final magnification of x 28,000. The values of AS were expressed as the mean number of granules per 1,000 nm length of GBM. The portion of the glomerular capillary wall, 7000 nm or more from the anchor portion was considered to be the peripheral portion. The length within 3,000 nm from the anchor portion was regarded as the proximal portion, and the portion of the basement membrane surrounding paramesangial area was considered to the paramesangial portion (Fig. 2). The mean number of PEI granules in each portion in two groups was estimated by Student's t-test for non-paired observations. Any cited difference is significant at the 5 % level or less.
RESULTS

SPECIFICITY OF THE ANTISERA FOR GBM

The AGBM was shown to react with glomerular capillary wall of normal dog kidney in a linear pattern (Fig. 1). Positive reaction was not observed in tubular basement membrane and vascular basement membrane in the interstitium.

URIALYSIS

Results were summarized in Fig. 3. Severe or mild proteinuria developed as early as day 1 and continued until week 2. The degree of proteinuria peaked on day 3 and thereafter decreased gradually. At weeks 4 and 8, there was no significant difference in the urinary protein between AGBM-treated group and NRS-treated group.

ULTRASTRUCTURAL STUDY FOR AS

Electron-dense granules of PEI were distributed regularly in the LRE of the GBM. However, the AS were distributed irregularly in the LRI and much
less in the number than those seen within the LRE (Fig. 4a). The number of AS in LRE of all three GBM portions was significantly low in AGBM-treated group as compared with NRS-treated group on weeks 1 and 2. The AS in the LRE at the portions with fusion of epithelial foot processes were comparable to those in the LRE without fusion of epithelial foot processes. On week 4, that in the peripheral portion was higher in AGBM-treated group than in NRS-treated group (p<0.05). No significant difference was seen in the number of AS in the LRE at the proximal and paramesangial portions on week 4 and any portions on week 8 between AGBM-treated group and NRS-treated group. The number of PEI granules of the LRI in both the peripheral and proximal portions also significantly decreased in AGBM-treated group on week 1. There were no significant differences in the number of granules in the peripheral and proximal portions on weeks 2, 4, and 8 (Tables 2 and 3, Figs. 4b-4d).

The ratio of the number of PEI granules in each portion in AGBM-treated group to that in NRS-treated group on weeks 1, 2, 4, and 8, was calculated (Figs. 5 and 6). The number of PEI
granules per unit length of the LRE or LRI was decrease in AGBM-treated group on weeks 1 and 2. The degree of decrease was more prominent on week 1 than that on week 2. But the degree of decrease of AS on LRE was similar in the peripheral, proximal, and paramelesangial portions. On week 4, a slight increasing of AS was noted in the number of PEI granules of the LRE in the peripheral portion. On week 4, in other portions, and week 8, in all portions, no changes in the ratio of the number of PEI granules of LRE or LRI were observed.

PATHOLOGICAL EXAMINATION

LIGHT MICROSCOPY

Intense swelling and hypercellularity of the glomeruli resulting in occlusion of the urinary space and capillary lumens was seen in the AGBM-treated dogs at week 1. Extensive necrosis with fibrinous deposits in the glomerulus was detected in extra- and intra-capillary sites (Fig. 7). Proliferation of parietal epithelium resulting in crescent formation was occurred in most glomeruli. In some glomeruli, the capillary tuft adhered to
Bowman's capsule. On week 2, these changes in the glomeruli were less severe. By week 4, glomerular necrosis had mostly disappeared. Most glomeruli showed the slight swelling, however there was a distinct proliferative of mesangial cells. At week 8, mesangial proliferation was detected in most glomeruli, and some glomeruli showed crescent formation, sclerosis with capsular adhesions and periglomerular fibrosis (Fig. 8). By immunoperoxidase method, the staining of HSPG on the GBM in AGBM-treated dogs was comparable to that in NRS-treated dogs on weeks 1, 2, 4, and 8 (Figs. 9a and 9b).

**IMMUNOFLUORESCENT TEST**

Striking linear deposition of rabbit IgG was found along the glomerular capillary walls of AGBM-treated dogs on week 1. On week 2, the linear deposition was still observed, but fluorescence was less well defined. By week 4, deposition of rabbit IgG was still remained. At week 8, a faint trace linear fluorescence for rabbit IgG was localized in the glomeruli (Figs. 10a-10d). At week 1, fluorescence for dog IgG was less bright, with a
focal segmental distribution. At week 2, the fluorescence, in a discontinuous linear fashion, was often focally distributed within the glomeruli. At weeks 4 and 8, these depositions were sharply defined; most glomeruli were brightly stained (Figs. 11a-11d). Discontinuous linear and focal granular deposition of dog C3 was detected along the glomerular capillary walls at week 1 (Fig. 12a). At week 2, these depositions were sharply defined, but the fluorescence had disappeared at week 4 and 8 (Fig. 12b).

ELECTRON MICROSCOPY

On week 1 postinjection, the GBM was irregularly swollen and sometimes laminated. The swollen GBM sometimes showed areas of a loose texture and fusion of epithelial foot processes. In the swollen GBM, the LRI distended to the subendothelial space (Fig. 13). Endothelial, epithelial and mesangial cells were swollen. In addition, granular, or fibrillar, masses of fibrin completely occluded the capillaries. On weeks 2, 4, and 8, although swollen GBM and distinct fusion of epithelial foot processes were still present, the severity of these
 ultrastructural changes tended to be less. In addition, the irregular thickening of GBM was detected on week 8. From week 4, mesangial proliferation and subepithelial dense deposits were characteristic (Fig. 14). On week 8, in some glomeruli, the mesangial area was still hypercellular with excessive amounts of matrix.
DISCUSSION

In our study, administration of anti-GBM serum resulted in glomerulonephritis with the onset of proteinuria. From one week after anti-GBM serum injection, the autologous phase of the glomerulonephritis was characterised by the deposition of host IgG. At this time, there were extensive glomerular necrosis, swelling and hypercellularity, crescent formation, and capsular adhesion. These lesions were succeeded by mesangial cell proliferation and glomerular sclerosis. These changes were in accord with those nephrotoxic glomerulonephritis (4, 5, 6, 7, 10, 12, 16, 17, 23, 25, 28, 33) including those in dogs (31, 44).

In AGBM-treated dogs with the severe or mild proteinuria, a loss of glomerular AS was evidenced. Thus, the proteinuria we observed in this study was associated with a decrease in AS in GBM, as have been noted in other renal disease (1, 3, 22, 27, 29, 34, 40, 42). In contrast, in rat model of puromycin aminonucleoside (PAN) nephrosis, AS distributions in GBM did not change significantly (9). Hence, it has been suggested that the proteinuria of disease is not related to the decreased AS of GBM (9, 19,
The differences in these results might have a possible explanation. Various cationic probes have been used to stain glomerular AS, including ruthenium red and cationic ferritin (14) and PEI (30). Potential differences in penetrability of polymers and small molecules with different isoelectric points, the method of administering the probe, and the incubation time in the probe have complicated interpretation of results obtained in different laboratories (9, 19, 20, 26, 36, 41).

In our study, on weeks 4 and 8, AGBM-treated dogs showed the improvement of proteinuria associating with recovery of AS in the GBM. The recovery of AS in the GBM were observed diffusely in peripheral, proximal, and paramesangial portions. On the other hand, in idiopathic membranous glomerulonephropathy proteinuria might result from reduction of AS, but in the later stage (Stage III) the recovery of AS was focal and so the improvement of proteinuria was not observed (45). These findings suggest that the improvement of proteinuria requires the diffuse and global recovery of AS in the GBM.

The reduction in staining of glomerular polyanion and swelling and lamination of GBM occurred simultaneously, in AGBM-treated dogs with severe or
mild proteinuria. These events obviously indicate the disturbance in the size and charge selective barriers of the GBM. But the question of which barriers may be first disrupted has not been resolved so far. The AS change may be the major of the proteinuria observed in autologous phase of canine AGBM nephritis, since in this study the degree of a reduction of glomerular AS was associated with the severity of proteinuria.

Loss of glomerular AS and fusion of epithelial foot processes are often seen in the glomerulonephritis with proteinuria. It is not clear in the relationship of epithelial foot process loss and reduction of AS in GBM. In this study, AS in the LRE of GBM with or without fusion of epithelial foot processes were quantitated separately. No significant difference in the number of AS in the LRE in these two portions. Thus, the reduction of AS in GBM in this model was not associated with fusion of epithelial foot processes.

An improvement of proteinuria was observed in AGBM-treated dogs even through fusion of epithelial foot processes and mesangial cell proliferations persisted. A similar phenomenon was observed in the aminonucleoside nephritis (1). The increased
glomerular permeability in these models appears not to be associated with fusion of epithelial foot processes and mesangial cell proliferations.

HSPG is the major component of the AS in GBM (13, 14, 15, 41). In this study, a marked loss of AS was observed on week 1 postinjection. However, there was no significant difference in the intensity of HSPG-immunostaining between AGBM-treated and NRS-treated groups. It suggests that there might be no significant changes in HSPG content of GBM in AGBM-treated dogs. In PAN nephrosis, HSPG has not shown significant immunohistochemical alterations (20, 35). On the other hand, in PAN nephrosis, an immunohistochemical loss of HSPG has been implicated in the pathogenesis of the nephrotic syndrome (22).

The non-congruency of these results can be discussed from many aspects. First of all, epitopes towards which anti-HSPG antibody for immunohistochemistry is directed may be different. As another reason for the discordance, nephrotic serum neutralizes the charge of HSPG and diminishes the affinity of PEI without decreasing total HSPG content. But this explanation would be denied because on weeks 4 and 8, the deposits (rabbit IgG or dog IgG) were obviously detected in the
capillary walls or GBM, but a reduction of glomerular AS was not found in AGBM-treated dogs. In addition, elucidation of the correlation between glomerular AS and glomeruli HSPG in nephrotic syndrome must await the investigations about the degree of sulfation or molecular structure of HSPG.

In our study, AGBM-treated dogs showed a higher count of AS in the LRE as compared with control animals. In some proteinuric states, such as passive Hymann nephritis, increased numbers of AS have been observed between podocyte slit pores and immune complexes (32). This finding was interpreted as regeneration of AS in the GBM in response to immunologic injury. Injury to blood vessels (i.e., experimental deendothelization) also produced an increased number of AS during repair (43). In nephrotic serum nephritis, AS were demonstrable in the split regions of lamina densa with focal foot process fusion, about 24 hours after injury (25). Their appearance coincided with decreasing of the urinary protein level suggesting a repair phenomenon. Our studies suggest the regeneration of AS in the GBM coincide with an improvement of proteinuria in canine anti-GBM nephritis. On the other hand, in various human renal diseases, AS in
the lamina densa (LD) have been found to increase with the level of proteinuria (11), and this may reflect the unmasking of hidden AS. The multilaminar splitting of GBM in Samoyed dogs may permit greater penetration of PEI, resulting in the staining of AS which hidden in normal GBM (36). Possibly, hidden AS might have been exposed in GBM in AGBM-treated dogs. In some instance, negatively charged serum proteins might have been trapped in abnormal GBM in this AGBM nephritis resulting in AS increasing shown in human glomerulonephritis (11). As in GBM have been shown to contain HSPG by both enzymatic digestion studies (14, 41). These enzyme digestion studies would be helpful to determine which (if any) proteoglycan was contained in these AS.

Increased number of glomerular AS in the LRE was shown in parallel with that subepithelial deposits which were enclosed by newly synthesized basement membrane materials in this study. A similar phenomena is well document in human membranous glomerulonephritis (8, 38). Törnroth et al suggested some macromolecular substances including immune deposits or serum protein, due to the hyperpermeability of GBM, might make contact with the epithelial cells and its stimuli accelerate the
synthesis of basement membrane materials (37, 39). Hence, the possibility exists that the subepithelial deposits stimulate the synthesis of the GBM materials. This step will play a more important role in recovering the AS in GBM.

In summary, we have studied the structural and functional alterations of the autologous phase of canine anti-GBM nephritis. The fact that a reduction of glomerular AS occurred in AGBM-treated dogs with severe or mild proteinuria and the recovery of AS in the GBM coincided with an improvement of proteinuria suggested that alterations in glomerular AS might play an important role in the pathogenesis of proteinuria in the autologous phase of canine anti-GBM nephritis.
ACKNOWLEDGMENTS

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Table 1. Schedule for urialysis and pathological examinations.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>No. of animals for urialysis</th>
<th>No. of animals for pathological examination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NRS</td>
<td>AGBM</td>
</tr>
<tr>
<td>Dogs (Observed for 1 week)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
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</tr>
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<td>4</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>7 (1W)*</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Dogs (Observed for 8 weeks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3</td>
<td>4</td>
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<td>5</td>
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</tr>
<tr>
<td>7</td>
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<td>4</td>
</tr>
<tr>
<td>14 (2W)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>23</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>28 (4W)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>37</td>
<td>3^b</td>
<td>4</td>
</tr>
<tr>
<td>44</td>
<td>3^b</td>
<td>4</td>
</tr>
<tr>
<td>51</td>
<td>3^b</td>
<td>4</td>
</tr>
<tr>
<td>58 (8W)</td>
<td>3^b</td>
<td>4</td>
</tr>
</tbody>
</table>

NRS, Normal rabbit serum-treated dogs;  
AGBM, Anti-glomerular basement membrane-treated dogs;  
N, Not examined;  
*Week postinjection;  
^aOne dog is accidental death;  
^bAutopsy;  
^cBiopsy.
Table 2. Number of PEI granules per 1000 nm of the lamina rara externa (LRE) of glomerular basement membrane in each portion in NRS-treated and AGBM-treated Groups.

<table>
<thead>
<tr>
<th>Portion</th>
<th>Periods</th>
<th>1 week</th>
<th>2 week</th>
<th>4 week</th>
<th>8 week</th>
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<tr>
<td></td>
<td>NRS</td>
<td>AGBM</td>
<td>NRS</td>
<td>AGBM</td>
<td>NRS</td>
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<tr>
<td>Peri.</td>
<td>14.2±2.4</td>
<td>10.5±1.8</td>
<td>16.4±2.2</td>
<td>14.7±2.3</td>
<td>19.0±1.6</td>
</tr>
<tr>
<td>Pro.</td>
<td>15.0±1.4</td>
<td>10.8±1.9</td>
<td>17.3±3.2</td>
<td>14.9±2.3</td>
<td>19.7±2.3</td>
</tr>
<tr>
<td>Para.</td>
<td>13.4±2.1</td>
<td>8.4±1.8</td>
<td>17.0±3.3</td>
<td>13.8±2.3</td>
<td>19.6±1.7</td>
</tr>
</tbody>
</table>

Values, Mean±SD;
NRS, normal rabbit serum;
AGBM, anti-glomerular basement membrane serum;
Peri., Peripheral portion;
Pro., Proximal portion;
Para., Paramesangial portion;
*, p<0.05; **, p<0.01; ***, p<0.001 (Student's t-test).
Table 3. Number of PEI granules per 1000 nm of the lamina rara interna (LRI) of glomerular basement membrane in each portion in NRS-treated and AGBM-treated Groups.

<table>
<thead>
<tr>
<th>Portion</th>
<th>Periods:</th>
<th>1 week</th>
<th>2 week</th>
<th>4 week</th>
<th>8 week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group :</td>
<td>NRS</td>
<td>AGBM</td>
<td>NRS</td>
<td>AGBM</td>
</tr>
<tr>
<td>Peri.</td>
<td>5.5±1.4</td>
<td>5.0±1.3</td>
<td>*</td>
<td>7.6±1.7</td>
<td>7.2±1.8</td>
</tr>
<tr>
<td>Pro.</td>
<td>6.4±1.5</td>
<td>5.6±1.1</td>
<td>**</td>
<td>8.1±2.5</td>
<td>7.5±2.1</td>
</tr>
</tbody>
</table>

Values, Mean±SD;
NRS, normal rabbit serum;
AGBM, anti-glomerular basement membrane serum;
Peri., Peripheral portion;
Pro., Proximal portion;
Para., Paramesangial portion;
* , p<0.05; **, p<0.01 (Student's t test).
Fig. 1 Immunoperoxidase staining of the canine kidney with rabbit anti-canine glomerular basement membrane serum showing striking linear reaction along the glomerular capillary walls. x 360.

Fig. 2 Schema of the three portions of the basement membrane.

Peri., Peripheral portion: , arrowhead ~ long arrow ≥ 7,000 nm; Pro., Proximal portion: , short arrow ~ long arrow ≤ 3,000 nm; Para., Paramesangial portion; Ep, epithelial cell; Ed, endothelial cell; Mc, mesangial cell; *, Anchor portion.

Fig. 3 Urinary protein excretion per 15 h. Mean ± SD.

○, Normal rabbit serum-treated group; ●, Anti-glomerular basement membrane serum-treated group; *, p < 0.05; **, p < 0.01 (Wilcoxon rank sum test).
Fig. 4 Electron micrographs of glomerular basement membranes (GBM) from anti-GBM serum-treated dogs on weeks 1 (a), 2 (b), 4 (c), and 8 (d) postinjection (PEI staining). x 28,000
(a). Marked loss of PEI staining at the lamina rara externa (LRE) of the GBM (arrowheads).
(b). Loss of PEI staining at the LRE of the GBM (arrowhead) with lesser extent than in (a).
(c), (d). The GBM without any change in the distribution of anionia sites.

Fig. 5 Ratio of the number of PEI granules in the LRE of glomerular basement membrane (GBM) in anti-GBM serum (AGBM)-treated group to that in normal rabbit serum (NRS)-treated group (NRS: 100%). In the AGBM-treated group, ○, peripheral portion; □, proximal portion; △, paramesangial portion.
Fig. 6 Ratio of the number of PEI granules of the LRI of glomerular basement membrane (GBM) in anti-GBM serum (AGBM)-treated group to that in normal rabbit serum (NRS)-treated group (NRS: 100%). In the AGBM-treated group, ○, peripheral portion; □, proximal portion.

Fig. 7 Glomeruli from the anti-glomerular basement membrane serum-treated dog on week 1 postinjection. H.E. x 360.

Swollen glomeruli with hypercellularity, necrosis, epithelial crescent formation, and fibrinous deposits.

Fig. 8 Proliferative glomerulonephritis from the anti-glomerular basement membrane serum-treated dogs on week 8 postinjection. H.E. x 360.

Obsolescent glomerulus with periglomerular fibrosis (arrow).
Fig. 9 Immunoperoxidase staining with heparan sulfate proteoglycan (HSPG) in glomeruli obtained from normal rabbit serum-treated (a) and anti-glomerular basement membrane serum-treated (b) dogs on week 1 postinjection. x 360.

No significant qualitative differences in the immunoperoxidase reaction product over the glomerular capillary walls is shown.

Fig. 10 Photomicrographs of immunofluorescent test demonstrating rabbit immunoglobulins in the glomeruli obtained from weeks 1 (a), 2 (b), 4 (c), and 8 (d) of anti-glomerular basement membrane serum-treated dogs. x 720.
(a) Striking linear deposition along the glomerular capillary walls.
(b) Linear deposition along the glomerular capillary walls with lesser extent than in (a).
(c) The pattern of fluorescence is still linear.
(d) Traces linear fluorescence in the glomerulus.
Fig. 11 Photomicrographs of immunofluorescent test demonstrating dog immunogloburins in the glomeruli obtained from weeks 1 (a), 2 (b), 4 (c), and 8 (d) of anti-glomerular basement membrane serum-treated dogs. x 720.

(a) Fine granular staining along the glomerular capillary walls.

(b) Linear deposition along the glomerular capillary walls.

(c), (d) Striking linear deposition along the glomerular capillary walls.

Fig. 12 Photomicrographs demonstrating dog C3 by direct immunofluorescence in glomeruli from weeks 1 (a) and 2(b) anti-glomerular basement membrane serum-treated dogs. x 720.

(a) Faint linear deposition along the glomerular capillary walls.

(b) Linear deposition along the glomerular capillary walls.
Fig. 13 Electron micrograph of glomerulus from anti-glomerular basement membrane serum-treated dogs on week 1 postinjection. x 28,000. Distended lamina rara interna with less electron dense and fusion of epithelial foot processes.

Fig. 14 Electron micrograph of glomerular capillary tuft from anti-glomerular basement membrane (GBM) serum-treated dogs on week 8 postinjection. x 28,000. Irregular thickening of GBM with subepithelial deposits and fusion of epithelial foot processes.
Fig. 1
Fig. 2

Diagram showing anatomical structures with labels:
- Ep
- Ed
- Pro.
- Mc
- Peri.
- Para.
Fig. 3

*, P<0.05
Fig. 6

NRS: 100%
Fig. 12

(a)

(b)
Fig. 13
Fig. 14