

ANTIBODIES TO BASEMENT MEMBRANE HEPARAN  
SULFATE PROTEOGLYCANS BIND TO THE LAMINAE  
RARAE OF THE GLOMERULAR BASEMENT MEMBRANE  
(GBM) AND INDUCE SUBEPITHELIAL GBM THICKENING

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Heparan sulfate proteoglycans (HSPG)<sup>1</sup> are important components of the glomerular basement membrane (GBM) (1, 2). Evidence has been obtained that the integrity of HSPG is essential for maintaining both the charge and size-selective properties of the GBM (1–3), and disturbances in glomerular HSPG have been described in association with several glomerular diseases (3–5). Moreover, autoantibodies against HSPG have been detected in the sera of patients with poststreptococcal glomerulonephritis (6).

Recently we have raised a polyclonal antibody [anti-HSPG(GBM)] that specifically recognizes the core protein of a population of HSPG ( $M_r = 130,000$ ) found in the GBM (7, 8) and in other renal basement membranes (5, 8), and we have shown that these antibodies bind to the laminae rarae of the rat GBM after intravenous injection (8). The findings showed that the core proteins of these HSPG, like their anionic glycosaminoglycan side chains (9, 10), are exposed to circulating macromolecules in the glomerulus, and they raised the possibility that HSPG might be involved in the pathogenesis of autoimmune diseases affecting glomeruli.

In this study we have injected anti-HSPG(GBM) IgG into rats and have determined the pathologic consequences of antibody binding to the GBM at selected intervals up to 2 mo thereafter. We were particularly interested in determining whether binding of antibodies to fixed, endogenous antigens concentrated in the laminae rarae of the GBM would lead to an immune complex-type of nephropathy with accumulation of subepithelial and/or subendothelial immune deposits (11), or cause an anti-basement membrane type of glomerular disease with uniform binding of antibodies in a linear pattern to the GBM (12). We report here our pathophysiologic, light microscopic, and electron microscopic findings indicating that binding of anti-HSPG(GBM) IgG induces an anti-basement membrane type of disease with proteinuria and subepithelial basement

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<sup>1</sup>Abbreviations used in this paper: GBM, glomerular basement membrane; HRP, horseradish peroxidase; HSPG, heparan sulfate proteoglycan(s); LRE, lamina rara externa; LRI, lamina rara interna; PLP, periodate-lysine-paraformaldehyde fixative; PMN, polymorphonuclear leukocyte(s).

membrane thickening. An abstract describing these results has also been published (13).

### Materials and Methods

**Anti-Heparan Sulfate Proteoglycan Antibodies.** Rabbit anti-HSPG(GBM) was characterized in detail elsewhere (8). In brief, the antiserum was raised against proteoglycans purified from isolated rat glomeruli by ion exchange chromatography. It specifically recognizes the core protein of the  $M_r = 130,000$  basement membrane HSPG, does not show crossreactivity with laminin, type IV collagen, or fibronectin by ELISA, and selectively stains all basement membranes in rat kidney and other tissues by immunofluorescence and immunoperoxidase techniques (8). Rabbit antibodies against the Heymann nephritis antigen (gp330) (14) and a normal rabbit serum pool were used as controls.

IgG fractions were isolated from the rabbit sera by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ), and the IgG content of the fractions was determined by measuring the OD at 280 nm. The anti-HSPG(GBM) antiserum was absorbed with rat blood cells before isolation of IgG to remove crossreacting antibodies (15).

**Experimental Protocol.** Male Sprague-Dawley rats (Camm Research Lab Animals, Wayne, NJ, or Charles River Breeding Laboratories, Inc., Wilmington, MA), weighing 150–200 g, were injected intravenously via the external jugular vein with anti-HSPG(GBM) IgG (3–20 mg), or anti-gp330 IgG (5 mg), or normal rabbit IgG (21 mg) in 1 ml of PBS, pH 7.2. 22 rats received anti-HSPG(GBM) IgG, 6 rats received anti-gp330 IgG, and 3 rats received normal rabbit IgG.

Serum and urine samples were collected from experimental rats and from age-matched controls before, and at regular intervals of 3 min to 2 mo after, injection of rabbit IgG. Anti-HSPG(GBM) that had been preabsorbed with rat blood cells (15) was tolerated without incidence; the injected rats appeared normal during the 2-mo observation period, and no gross abnormalities were detected in their kidneys. If native (unabsorbed) IgG was given, some of the rats developed anaphylactic symptoms (respiratory distress), and one rat injected with 20 mg of IgG died immediately after injection.

**Preparation and Processing of Tissues for Immunocytochemistry and Electron Microscopy.** Tissue blocks were taken from the right kidneys, livers, spleens, and lungs of normal and experimental rats, snap frozen in isopentane cooled with liquid nitrogen, and stored in liquid nitrogen. Cryostat sections (5  $\mu\text{m}$ ) were prepared from unfixed tissues, mounted on glass slides, fixed in acetone for 10 min at  $-20^\circ\text{C}$ , and used for immunofluorescence staining.

The left kidney was flushed with PBS and fixed by retrograde perfusion via the abdominal aorta with a 2% solution of paraformaldehyde freshly made in a sodium phosphate buffer (0.04 M) containing lysine (0.08 M) and sodium metaperiodate (0.01 M) (16), after which tissue pieces were prepared and immersed in this fixative (PLP) for an additional 4 h at  $20^\circ\text{C}$ . Some of the tissue pieces were cryoprotected by incubation in 2.3 M sucrose, frozen in liquid nitrogen, and sectioned at 0.5  $\mu\text{m}$  on a Reichert Ultracut ultramicrotome (Reichert Scientific Instruments, Buffalo, NY) equipped with cryo-attachment using the techniques of Tokuyasu et al. (17). These semithin sections were then mounted on poly-L-lysine-coated slides and used for immunofluorescence. Other pieces of aldehyde-fixed kidney tissue were washed in PBS for 10 min, cryoprotected in 10% DMSO, snap frozen, and stored in liquid nitrogen until used for immunoperoxidase localizations as described below.

Tissue blocks were taken from both kidneys, immersed in 1% glutaraldehyde/4% formaldehyde in 0.1 M sodium cacodylate buffer for 1 h at  $20^\circ\text{C}$  or overnight at  $4^\circ\text{C}$ , postfixed in veronal acetate-buffered  $\text{OsO}_4$ , stained in block with uranyl acetate, and dehydrated and embedded in Epon for electron microscopy.

**Localization of Basement Membrane HSPG in Rat Kidney by Indirect Immunofluorescence.** Acetone-fixed cryostat sections (5  $\mu\text{m}$ ) or semithin frozen sections (0.5  $\mu\text{m}$ ) prepared from aldehyde-fixed tissue were incubated for 30 min to 2 h at  $20^\circ\text{C}$  with the

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anti-HSPG(GBM) IgG. After washing in PBS for 10 min, they were incubated with rhodamine-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) for 30 min, washed again in PBS, mounted in PBS-glycerol, pH 7.2, and examined in a photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) equipped with phase contrast and epifluorescence optics and appropriate filters for rhodamine and fluorescein.

*Detection of Anti-HSPG(GBM) IgG, Rat IgG, and C3 in Rat Tissues by Direct Immunofluorescence.* Cryostat sections were incubated with either rhodamine-labeled goat anti-rabbit IgG, fluorescein-labeled rabbit anti-rat IgG, or fluorescein-labeled goat anti-rat C3 antibodies (Cappel Laboratories) for 30 min at 20°C, washed in PBS, mounted with PBS-glycerol, and studied by immunofluorescence microscopy.

*Immunoperoxidase Staining Procedures.* Cryostat sections (20–30  $\mu\text{m}$ ) were prepared from PLP-fixed kidney tissues and stained by a direct immunoperoxidase technique based on an indirect immunoperoxidase procedure previously described (18). Briefly, the sections were incubated for 2 h at 25°C with either Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Biosys, Paris, France) to detect rabbit IgG, or with a sheep anti-rat C3 conjugated to HRP (Cappel Laboratories) to detect rat C3 bound to glomeruli. The sections were then fixed in 1.5% glutaraldehyde, incubated in diaminobenzidine medium containing 0.005%  $\text{H}_2\text{O}_2$ , postfixed in ferrocyanide-reduced  $\text{OsO}_4$ , and dehydrated and embedded in epoxy resin for electron microscopy. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope operating at 60 kV.

*Counts of Leukocytes Adhering to Glomeruli.* The number of polymorphonuclear leukocytes (PMN) per glomerulus was counted on semithin (0.5  $\mu\text{m}$ ) sections cut from Epon-embedded cryostat sections of rat kidney stained for rabbit IgG by the direct immunoperoxidase technique. At least three different glomeruli were counted from each kidney by phase contrast microscopy. Since the kidneys were perfused free of blood before fixation, only those leukocytes adhering to glomerular components remained.

*Detection of Rabbit Antibodies in Rat Sera.* Disappearance of the injected rabbit anti-HSPG(GBM) and anti-gp330 IgG from rat sera was monitored by an indirect immunofluorescence assay. Serial dilutions of serum samples obtained from 15 to 2 mo after antibody injection were applied to unfixed cryostat sections (5  $\mu\text{m}$ ) of normal rat kidney for 30 min. After washing in PBS the sections were incubated with rhodamine-labeled anti-rabbit IgG (Cappel Laboratories), washed again, and mounted for immunofluorescence microscopy. The staining of basement membranes or proximal tubular brush borders at serial dilutions was determined and used to assess the amounts of circulating anti-HSPG(GBM) or anti-gp330 IgG, respectively, remaining in the circulation. Rat sera were screened for the presence of anti-GBM autoantibodies by a similar immunofluorescence assay using fluorescein-labeled anti-rat IgG (Cappel Laboratories) as the second antibody.

*Detection of Anti-Rabbit IgG in Rat Sera.* The appearance of antibodies against rabbit IgG in the sera of rats injected with rabbit IgG was determined using double immunodiffusion in 1% agarose and an ELISA. For double diffusion, undiluted rat sera were reacted against normal rabbit IgG (1 mg/ml of PBS). For the ELISA, flat-bottomed microtiter wells (Nunc-immunoplate I F, Gibco Laboratories, Grand Island, NY) were coated with normal rabbit IgG (2.5  $\mu\text{g}/\text{ml}$ ), washed with PBS containing 0.05% Triton X-100, and quenched with 0.4% of BSA. They were then incubated with rat sera diluted in the same buffer followed by affinity-purified HRP-labeled goat anti-rat IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) and orthophenylenediamine substrate (Sigma Chemical Co., St. Louis, MO), and absorbance was read on a Titertek Multiskan microplate reader (Flow Laboratories, Inc., McLean, VA) (19). The conjugate was preabsorbed against rabbit IgG.

*Measurement of Urinary Albumin.* Urine samples were collected, usually for a period of 18 h, from rats kept individually in metabolic cages; they had free access to water. Samples were centrifuged in a table top centrifuge and stored at  $-20^\circ$  until albumin was measured by radial immunodiffusion in 1% agarose prepared in 0.2 M phosphate buffer, pH 8.4 (20), containing rabbit anti-rat albumin antibodies (Cappel Laboratories). Rat serum

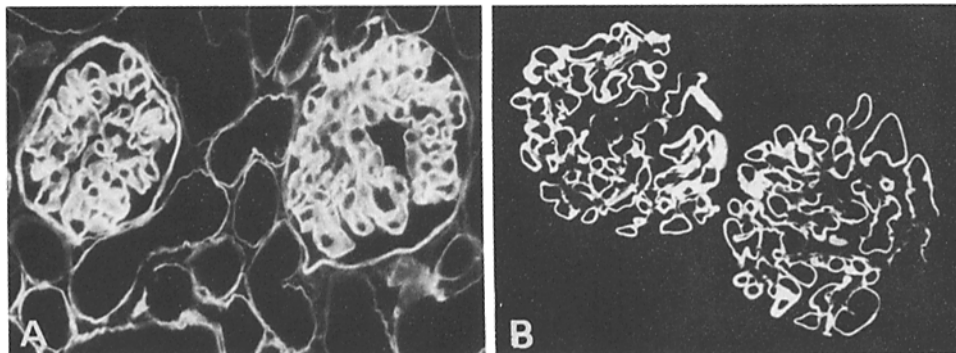


FIGURE 1. Fluorescence micrographs of semithin (0.5  $\mu\text{m}$ ) frozen sections of perfusion-fixed rat kidneys. (A) Section from a normal rat kidney stained by indirect immunofluorescence with anti-HSPG(GBM). The antibody stains all renal basement membranes, i.e., the GBM, Bowman's capsule, the basement membranes of renal tubules, and those of peritubular capillaries. (B) Direct immunofluorescence staining of the kidney of a rat at 2 h after intravenous injection of anti-HSPG(GBM) IgG. The antibody binds only to the GBM.  $\times 250$ .

albumin (Sigma Chemical Co.) was used as a standard. For statistical analysis the Student's *t* test was used.

**Quantitation of IgG Binding to Rat Tissues.** Anti-HSPG(GBM) and normal rabbit IgG were radiolabeled with  $^{125}\text{I}$  (Carrier free; Amersham Corp., Arlington Heights, IL) using Iodo-Beads (21) (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. The specific activities of the labeled antibodies varied from 0.8 to 2  $\mu\text{Ci}/\mu\text{g}$  IgG.  $^{125}\text{I}$ -Anti-HSPG(GBM) IgG (130–220  $\mu\text{g}$ ) or  $^{125}\text{I}$ -normal rabbit IgG (30–100  $\mu\text{g}$ ) were injected intravenously into three rats each, as described above. After 1–2 h the animals were perfused via the abdominal aorta with PBS, and the kidneys, lungs, livers, and spleens were removed, weighed, and counted for  $^{125}\text{I}$ . Glomeruli were prepared (22) from the kidneys of the rats killed 1 h postinjection and counted for bound  $^{125}\text{I}$ .

## Results

**Localization of Basement Membrane HSPG in Normal Rat Kidney by Indirect Immunofluorescence.** As reported previously (8), when the rabbit anti-HSPG(GBM) IgG was used to stain kidney sections by indirect immunofluorescence, it reacted with all renal basement membranes: the GBM, Bowman's capsule, and all tubular and vascular basement membranes (Fig. 1A). Rabbit antibodies against gp330, the pathogenic antigen of Heymann nephritis, bound to the brush borders of renal proximal tubules and gave a faint granular staining of glomeruli (22). Normal rabbit IgG did not stain any kidney structure.

**Rapid Depletion of Anti-HSPG(GBM) IgG from Rat Blood.** 15 min after intravenous injection of 5 mg of anti-HSPG(GBM) IgG, the injected IgG was detected in rat sera at a 1:1,000 dilution by the indirect immunofluorescence assay, at 2 h it was detected only in undiluted sera, and at 1 d none was found. These results show that the anti-HSPG(GBM) IgG rapidly disappeared from the sera of the injected rats. By contrast the anti-gp330 IgG disappeared only slowly from the circulation; at 15 min it was detected at a dilution of 1:100, after 1 d at 1:10, and at 2 wk was still detected in undiluted sera.

**Anti-HSPG(GBM) Binds Rapidly to the Laminae Rarae of the GBM.** As early as 3 min after intravenous injection of anti-HSPG(GBM) IgG, rabbit IgG was detected by light microscopy (Figs. 1B and 2A) as linear deposits outlining the

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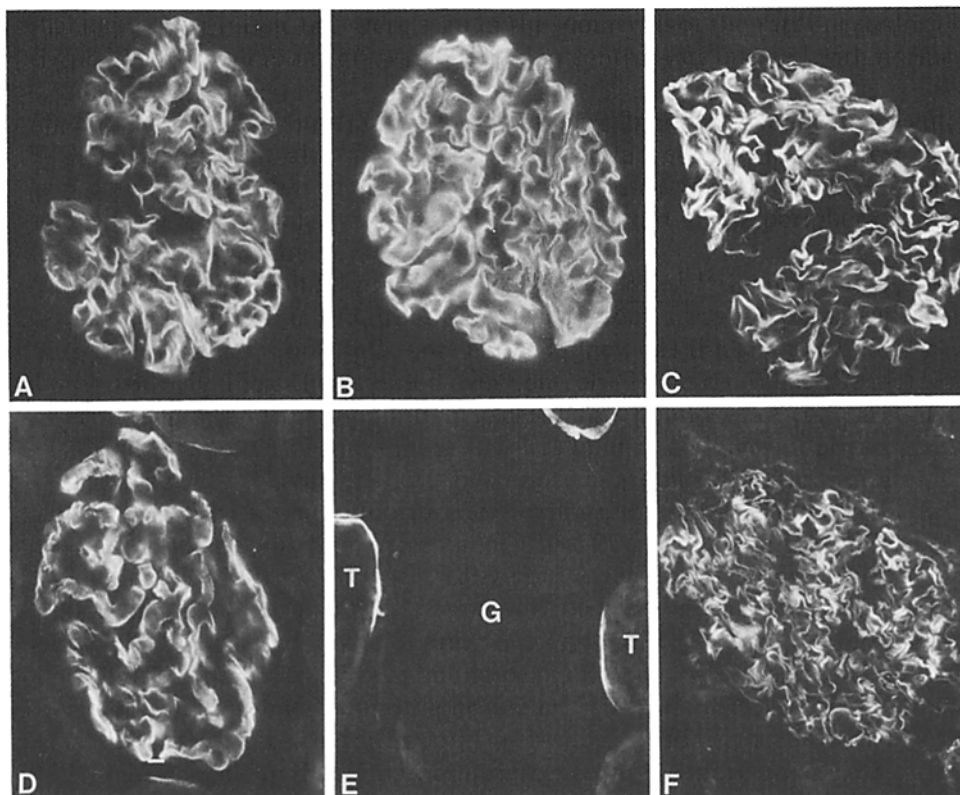


FIGURE 2. Immunofluorescence micrographs showing anti-HSPG(GBM) IgG (A–C), rat C3 (D and E), and rat IgG (F) bound to glomeruli in kidneys of rats injected with anti-HSPG(GBM) IgG. After intravenous injection, bright linear deposits of rabbit IgG are present in glomerular capillaries at all intervals, i.e., 15 min (A), 4 d (B), or 2 mo (C) postinjection. Linear deposits of C3 are also seen along the GBM at 2 h postinjection (D), but could not be detected in glomeruli after 4 d (E). C3 was seen along tubular basement membranes and Bowman's capsule (D and E) in all the injected animals, including normal controls. From 1 wk to 2 mo, linear deposits of rat IgG could be detected in the glomeruli of some of the rats injected with anti-HSPG(GBM) IgG (F). G, glomerulus; T, tubule.  $\times 450$ .

capillary walls of all glomeruli. This staining pattern suggested that the rabbit IgG was bound to the GBM. The staining intensity increased up to 2 h postinjection but thereafter remained virtually unchanged during the entire 2-mo observation period (Fig. 2, B and C, Fig. 3A; Table I). There was little or no binding of rabbit IgG to any other renal structure except for occasional areas where there was binding to the basement membranes of peritubular capillaries and those of the adjacent tubules (Fig. 3B). Such sites were found in 4 of the 14 rats injected with small amounts (3–5 mg) and in all of the 6 rats injected with larger amounts (12–20 mg) of anti-HSPG(GBM) IgG. Presumably this occurred due to local leakage of anti-HSPG(GBM) IgG from peritubular capillaries. Rabbit IgG was never detected in Bowman's capsule or in the walls of renal arterioles in any of the injected rats.

When the bound anti-HSPG(GBM) IgG was localized by direct immunoperoxidase staining at the electron microscope level, it was seen as early as 3 min after

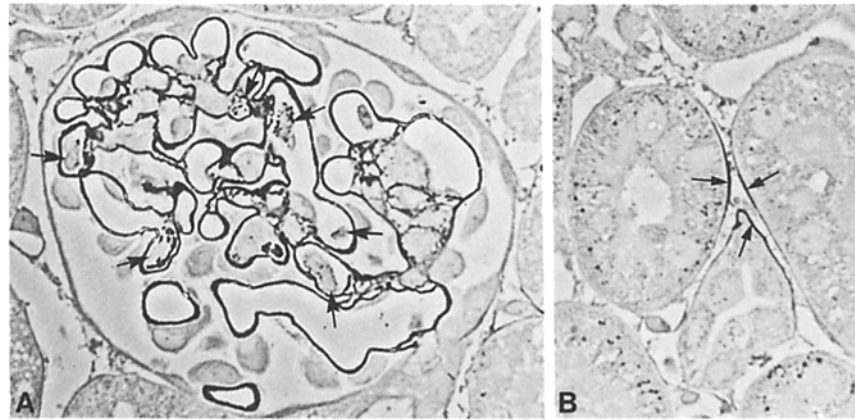


FIGURE 3. Phase contrast micrographs of semithin ( $0.5 \mu\text{m}$ ) sections cut from Epon-embedded cryostat sections of rat kidney stained for rabbit IgG by the direct immunoperoxidase technique. Sample taken from a rat given anti-HSPG(GBM) IgG 15 min before killing. (A) Rabbit IgG is deposited along the GBM and in mesangial areas. Several PMN leukocytes are present in the lumen of the glomerular capillaries (*arrows*). The PMN can be identified by their dense granules, which stain with the diaminobenzidine reagent due to their endogenous myeloperoxidase activity. (B) In some areas, rabbit IgG is detected in the basement membranes of a peritubular capillary and the immediately adjacent tubules (*arrows*).  $\times 800$ .

TABLE I  
*Immunofluorescence Findings in Kidneys of Rats after Intravenous Injection of Anti-HSPG(GBM)*

Time postinjection	Number of rats injected	Amount of IgG injected (mg)	Linear GBM deposits*		
			Rabbit IgG	Rat C3	Rat IgG
3 min to 1 d	7	3-5	++ (7/7) <sup>‡</sup>	++ (6/6)	- (0/6)
4 d	3	5-12	+++ (3/3)	± (2/3)	- (0/3)
1-3 wk	6	3-20	+++ (6/6) <sup>‡</sup>	± (1/5)	+ (4/5)
4-8 wk	6	5-20	+++ (6/6)	+ (4/6)	+ (6/6)

Semithin ( $0.5 \mu\text{m}$ ) or cryostat ( $5 \mu\text{m}$ ) sections of rat kidneys were incubated with fluorochrome-labeled antibodies against rabbit IgG, rat C3, and rat IgG.

\* +++, ++, +, ±, and - indicate very strong, strong, moderate, weak, and negative fluorescence after direct staining. Numbers in parentheses indicate number of samples positive per number of samples studied.

<sup>‡</sup> Includes a semithin section of a plastic-embedded cryostat section stained for rabbit IgG by the direct immunoperoxidase technique.

injection in the GBM and the mesangial matrix (Fig. 4). Within the GBM, binding occurred largely or exclusively to both laminae rarae, interna and externa, giving the GBM a tram-track appearance (Fig. 5, A and B). Little or no reaction product was seen in the lamina densa. At early intervals (3 min to 4 d), staining of the lamina rara interna (LRI) was somewhat more extensive than that of the lamina rara externa (LRE), whereas at later intervals (4 d to 1 mo) the situation was reversed, and binding to the LRE predominated. Staining of the mesangial matrix was most extensive at 1 d postinjection, after which it gradually disappeared.

In controls injected with anti-gp330 IgG, the staining pattern was quite

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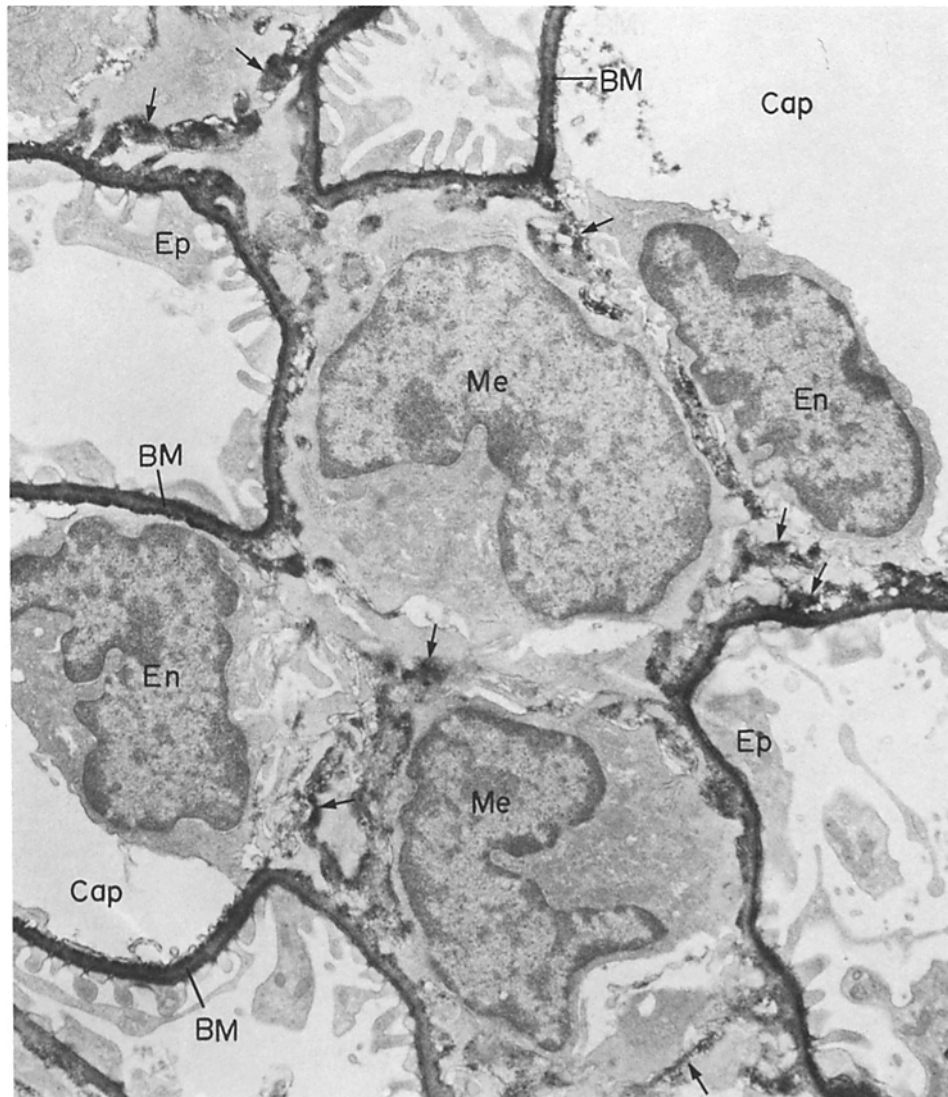


FIGURE 4. Electron micrograph of a field from the glomerulus of a rat injected with 20 mg anti-HSPG(GBM) IgG, 1 d before killing. The section was prepared from aldehyde-fixed kidney tissue, which has been stained by direct immunoperoxidase to localize rabbit IgG. Reaction product indicating the sites of deposited IgG, is detected in the mesangial matrix (*arrows*) and in the GBM (*BM*) where it is concentrated in the laminae rarae. There is no staining of the endothelial (*En*), epithelial (*Ep*), or mesangial (*Me*) cells. *Cap*, capillary lumen.  $\times 10,000$ .

different from that obtained with anti-HSPG(GBM) IgG, and binding to glomeruli increased gradually with time; at 15 min postinjection a faint granular fluorescence was seen along the glomerular capillary walls which became increasingly more intense by 2 h, and the size of the granular deposits increased up to 3 wk. In controls injected with 21 mg of normal rabbit IgG, a faint glomerular and interstitial staining for rabbit IgG was seen in rat kidneys 2 h after injection

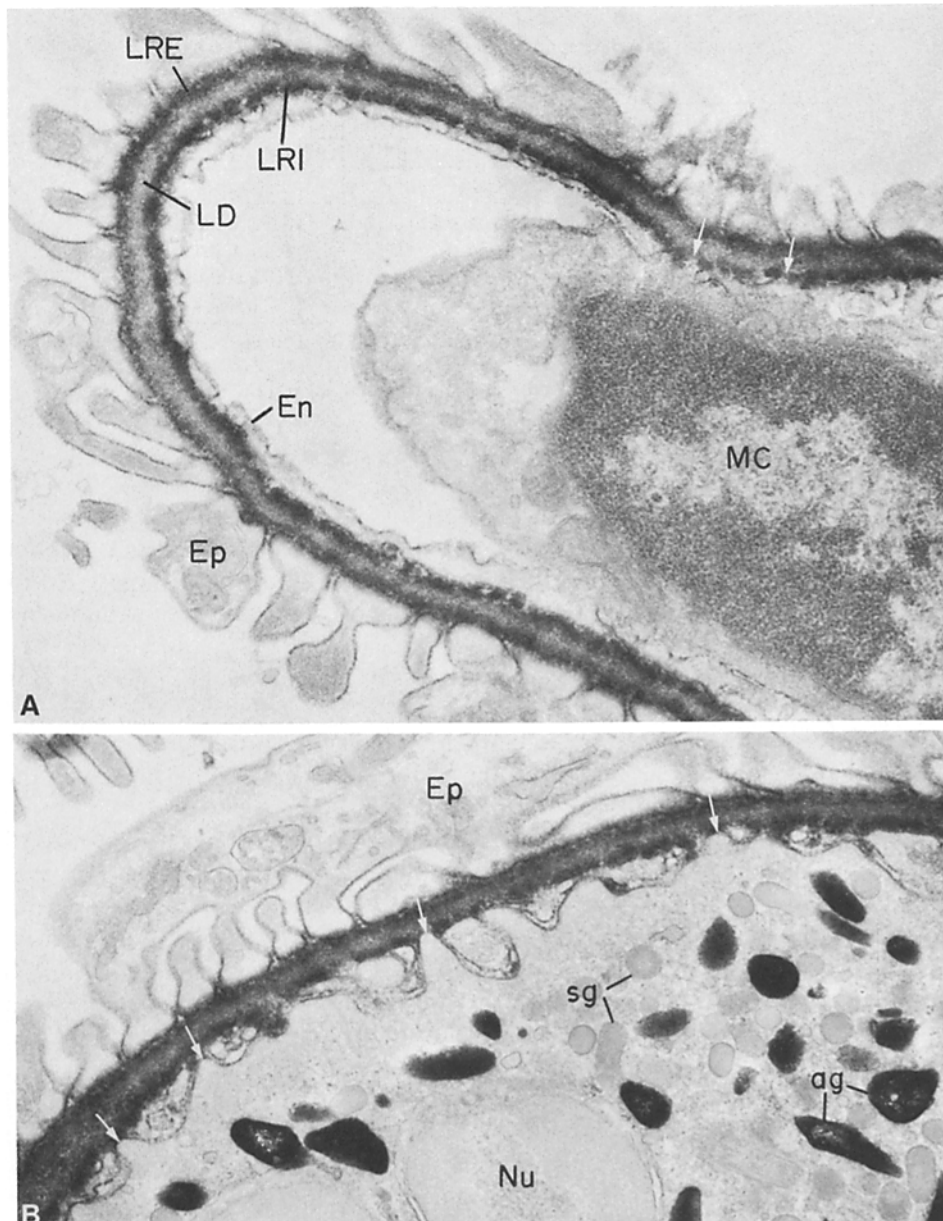


FIGURE 5. Preparations similar to that in Fig. 4, from a rat killed 1 d (A) and 2 h (B) after injection of anti-HSPG(GBM) IgG. At higher magnification the bound rabbit IgG is seen to be concentrated in the lamina rara interna (LRI) and lamina rara externa (LRE) of the GBM, giving the GBM a tram-track appearance. By contrast, there is little or no staining of the lamina densa (LD) in either field. The lumen of the capillary in A contains a mononuclear cell (MC), believed from its morphology to be a lymphocyte, and the lumen of the capillary shown in B contains a PMN, identified by its multilobed nucleus (nu) and characteristic specific (sg) and azurophil (ag) granules. Both cells can be seen to extend multiple pseudopods (arrows) through the endothelial fenestrae to directly contact the LRI of the GBM where the rabbit IgG and rat C3 (see Fig. 6) are deposited.  $\times 26,000$ .



TABLE II  
*Distribution of Radioactivity after Injection of <sup>125</sup>I-labeled Anti-HSPG(GBM) IgG in Rats*

Organs*	Percent injected radioactivity bound per gram tissue	
	Anti-HSPG(GBM)	Normal rabbit IgG
Kidneys (6)	0.63 ± 0.20 <sup>‡</sup>	0.27 ± 0.17
Lungs (3)	0.58 ± 0.25	0.46 ± 0.11
Livers (3)	0.36 ± 0.16	0.31 ± 0.09
Spleens (3)	0.47 ± 0.26	0.27 ± 0.12

130–220 µg of <sup>125</sup>I-labeled anti-HSPG(GBM) or 30–100 µg <sup>125</sup>I-labeled normal rabbit IgG were injected intravenously. 1 or 2 h later, the animals were killed, perfused via the abdominal aorta, and the organs were weighed and counted for radioactivity.

\* Numbers in parentheses indicate the number of organs counted.

<sup>‡</sup> Significantly increased binding of anti-HSPG(GBM) IgG as compared with normal rabbit IgG ( $p < 0.01$ ) was found only in kidney tissue.

but was not detectable thereafter. By immunoperoxidase staining only subepithelial deposits of rabbit IgG were seen in the glomeruli of the rats injected with anti-gp330 IgG. No rabbit IgG was detected in control rats injected with normal rat IgG.

*Binding of Injected Anti-HSPG(GBM) to Other Organs.* The injected anti-HSPG(GBM) IgG was also detected by immunofluorescence in the liver and spleen where it was seen in the walls of blood vessels and, in the case of the spleen, in the extracellular matrix of the red pulp and at the periphery of the white pulp (data not shown).

*Quantitation of the Bound Anti-HSPG(GBM) IgG.* When <sup>125</sup>I-anti-HSPG(GBM) IgG was injected into rats and the amount of IgG bound was compared with that of rats given normal rabbit IgG, only in the kidney was the amount bound per gram of tissue significantly increased over controls (Table II). When the glomeruli were isolated, and the amounts of labeled IgG bound were compared 1 h after injection, about 100 times more IgG was bound to glomeruli of rats given anti-HSPG(GBM) IgG than to those given normal rabbit IgG. This indicates, in confirmation of the immunofluorescence findings, that most of the specific IgG was bound to glomeruli. After injection of 130–220 µg of <sup>125</sup>I-anti-HSPG(GBM) IgG, ~0.36% of the IgG was specifically bound per gram kidney tissue (Table II). Assuming that the same percentage of total IgG given would bind after injection of 3, 5, 12, and 20 mg of unlabeled anti-HSPG(GBM) IgG, we estimate that ~11, 18, 43, and 72 µg were bound per gram kidney, respectively.

Liver, spleen, and lung bound slightly more <sup>125</sup>I-anti-HSPG(GBM) IgG than normal rabbit IgG, but the differences were not statistically significant (Table II). It can be concluded that immunofluorescence gives a more precise indication of the sites of deposition of specific IgG than do measurements of radioactivity because of the high background of radiolabeled nonspecific IgG.

*Rat C3 Binds Exclusively to the LRI.* By immunofluorescence, linear deposits of rat C3 were seen along the capillary walls of all glomeruli as early as 3 min after injection of anti-HSPG(GBM) IgG (Table I). The amount present increased

up to 2 h postinjection (Fig. 2D) but thereafter decreased rapidly, and C3 was no longer detectable in glomeruli at 4 d postinjection (Fig. 2E). From 1 wk postinjection on, deposits of C3 reappeared in the glomeruli of some of the rats (Table I). This occurred at the same time as rat antibodies against rabbit IgG appeared in the sera (see below) and linear deposits of rat IgG were first found in glomeruli of the injected rats (Fig. 2F; Table I). No binding of C3 to glomeruli was detected after injection of normal rabbit IgG. However, as reported by others (23), all rats, controls included, showed deposits of C3 along some tubular basement membranes (Fig. 2, D and E).

When C3 was localized at the electron microscope level by direct immunoperoxidase staining, at 3 min to 1 d it was seen to be bound to the GBM where it was confined exclusively to the LRI and to the mesangial matrix (Fig. 6). No staining of the lamina densa or LRE was seen. At 4 d, C3 was no longer detected in the LRI, but at 2 wk postinjection staining of the LRI reappeared in some glomeruli, in keeping with the immunofluorescence results.

*Early Morphological Changes Induced in the LRI by Binding of Anti-HSPG GBM.* In the early stages (15 min to 4 d) after injection of anti-(HSPG) IgG, the only morphological changes detected in the GBM were confined to the LRI; periodic, flocculent subendothelial deposits of moderate electron density were visible in routine morphologic preparations, and there appeared to be a general distention of the LRI (Fig. 7). The location of the deposits in the LRI corresponded to the sites of deposition of rabbit IgG and C3. A lifting or swelling of fenestrated portions of the endothelial cytoplasm was also evident in some places (Fig. 7). By 4 d these deposits were no longer visible and the swelling was no longer evident. Thus, the presence of morphologically recognizable, moderately dense deposits corresponded closely in time and location to that of rat C3.

*Adherence of Leukocytes to the GBM.* Increased numbers of mononuclear (Figs. 5A and 6) and PMN (Figs. 3A and 5B) leukocytes were seen adhering to the walls of many glomerular capillaries at intervals of 3 min to 4 d after injection of the anti-HSPG(GBM) IgG. They were often seen to extend pseudopods through the endothelial fenestrae to come into direct contact with the LRI (Figs. 5 and 6) where the rabbit IgG and the rat C3 deposits were located. The identity of the mononuclear cells could not be determined with certainty, but those that adhered during the first 4 d postinjection (Figs. 5A and 6) resembled lymphocytes in their morphology (rounded nuclei, high nuclear/cytoplasmic ratio), and those that bound at later times (1 wk to 2 mo) resembled more monocytes (crescentic nuclei, more abundant cytoplasm).

When PMN were counted by phase contrast microscopy in semithin sections (Fig. 3A), their number was found to be increased at all intervals up to 1 d postinjection. The average number of PMN per glomerulus at 8 min, 15 min, 2 h, and 1 d postinjection was 1.5, 4.5, 1.8, and 1.4, respectively. After 4 d the number decreased (0–0.8) and was comparable to that found in controls injected with normal rabbit IgG (0–0.3). The number of mononuclear leukocytes was not quantitated because they could not be distinguished from resident glomerular cells by light microscopy.

*Induction of Anti-Rabbit IgG.* Antibodies against rabbit IgG were detected in the sera of most rats after 1 wk postinjection. 9 out of the 11 anti-HSPG(GBM)

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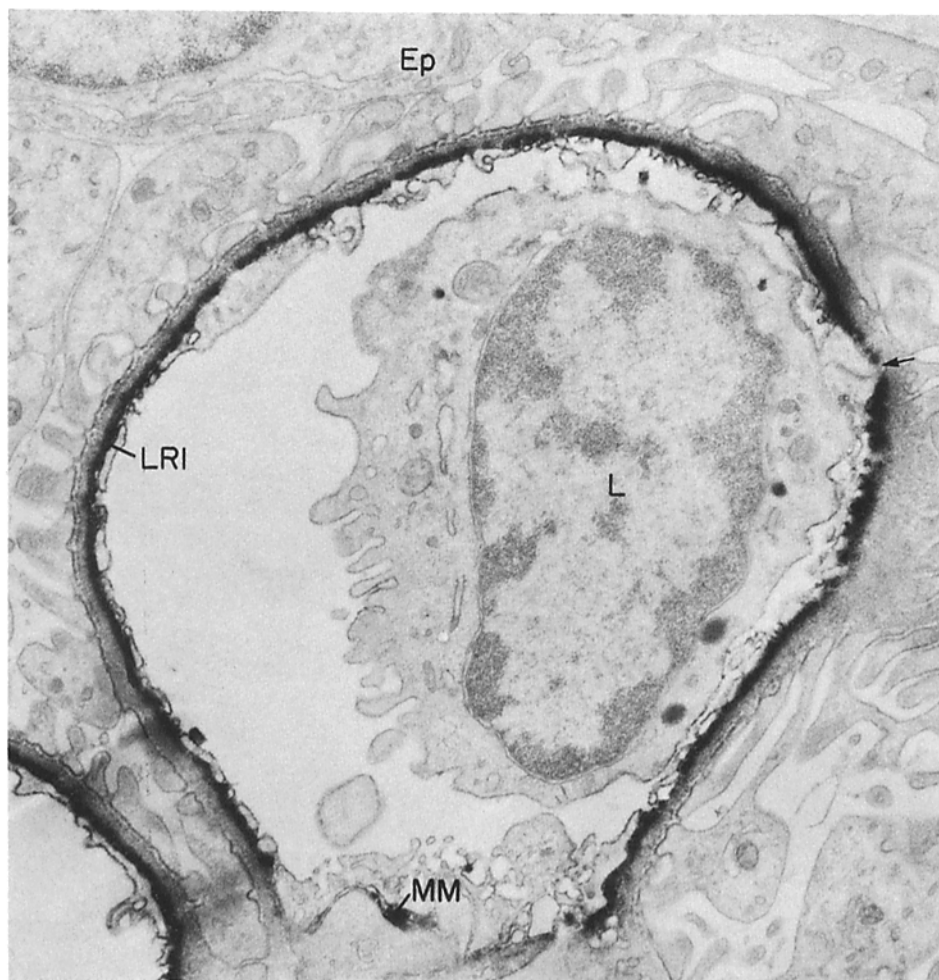


FIGURE 6. Field from a section prepared from the kidney of a rat killed 15 min after injection of anti-HSPG(GBM) IgG, which has been stained by direct immunoperoxidase for the presence of rat C3. C3 is found in the GBM and mesangial matrix (MM). It is localized exclusively in the LRI (LRI) of the GBM. A circulating cell, believed to be a lymphocyte (L), is seen to extend a pseudopod through an endothelial fenestra (arrow) to contact the LRI of the GBM.  $\times 14,000$ .

IgG-injected rats tested, and 2 out of the 4 controls injected with either anti-gp330 or normal rabbit IgG tested showed circulating antibodies with varying titers (1:10 to 1:1,000) by ELISA. No direct relationship between antibody titers and amount of albuminuria (see below) was apparent, and precipitating antibodies were not seen in the immunodiffusion assay.

No autoantibodies against the GBM or other kidney basement membranes were detected in the sera of rats injected with anti-HSPG(GBM) IgG by indirect immunofluorescence.

*Induction of Subepithelial Basement Membrane Thickening.* As early as 9 d after injection of anti-HSPG(GBM) IgG some thickening and distortion of the GBM

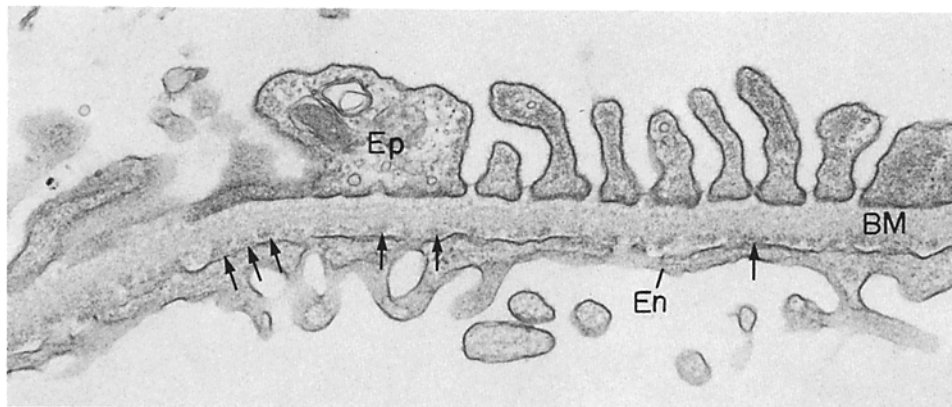


FIGURE 7. GBM from a rat injected with 5 mg anti-HSPG(GBM) 15 min before killing. The section was prepared from aldehyde-fixed kidney tissue which had been stained in block with uranyl acetate, and processed for routine morphology. Punctate electron-dense deposits are seen in the LRI (arrows). They correspond in their location to that of the immune deposits containing C3 (see Fig. 6). The LRI appears broadened and distended, displacing the endothelium (En) in places, while the LRE and lamina densa of the GBM appear normal.  $\times 30,000$ .

was noted, which varied in extent from loop to loop (Fig. 8). The GBM thickening was more prominent in rats that received 20 mg of IgG than in those that were given only 5 mg. It became progressively more severe with time, and by 2 mo postinjection some areas of the GBM were two to three times their normal width, and the outer aspect of the GBM often had a scalloped appearance (Figs. 8 and 9).

In specimens prepared for routine morphology, several morphologically distinct layers of basement membrane-like material could often be detected in the thickest regions of the GBM (Fig. 8B). In sections stained for rabbit IgG (Fig. 9), it was clearly seen that the thickening and scalloped appearance was due to the addition of an uneven layer of material with a density comparable to that of the lamina densa on the epithelial side of the original GBM, which displaced the bound IgG toward the endothelium (Fig. 9, B and C). In some regions the original LRI and LRE could still be detected (by virtue of the bound IgG) in the thickened basement membrane (Fig. 9B), but in others only the residual LRE and lamina densa were visible (Fig. 9C), suggesting that the IgG bound to the LRI (Fig. 9A) had been removed from the endothelial side or denatured.

No thickening of the GBM was observed at any interval (up to 2 mo) in age-matched controls given 20 mg of normal rabbit IgG.

**Increased Urinary Excretion of Albumin.** The binding of anti-HSPG(GBM) IgG to the GBM caused a moderate but significant increase in urinary albumin excretion (Fig. 10) that was clearly dose dependent. The mean albuminuria of rats injected with 12 mg of anti-HSPG(GBM) IgG or more was 1.2 (range 0.1 to 3.2) mg/24 h in the first 1–5 d after injection of rabbit IgG, and increased to 12.0 (range 6.6 to 17.2) mg/24 h by 2 mo postinjection. The mean excretion of albumin of 1- and 3-mo-old control rats were 0.4 (range 0.04 to 1.7) and 0.7 (range 0.2 to 1.9) mg/24 h, respectively. These results show that the binding of

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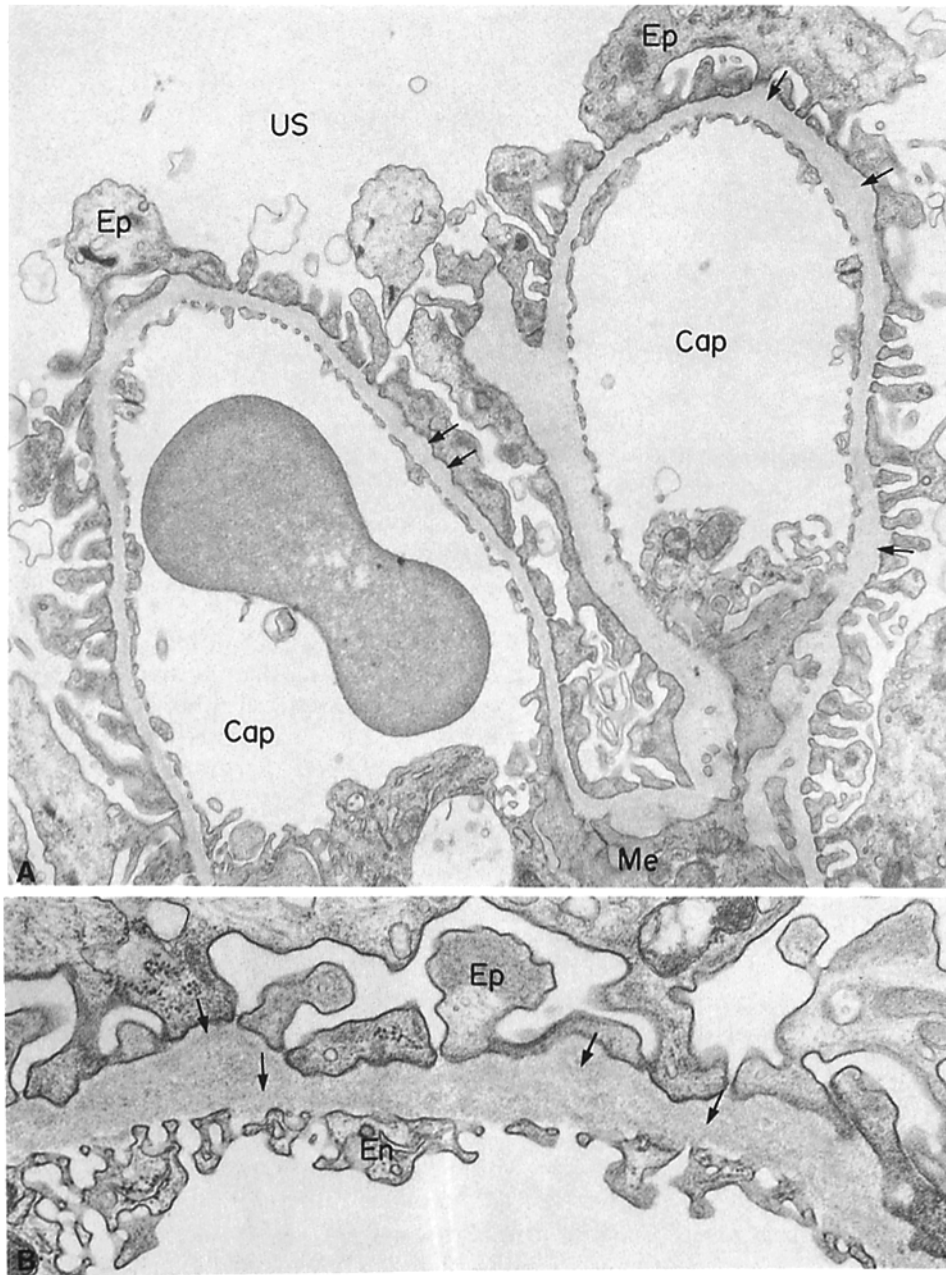
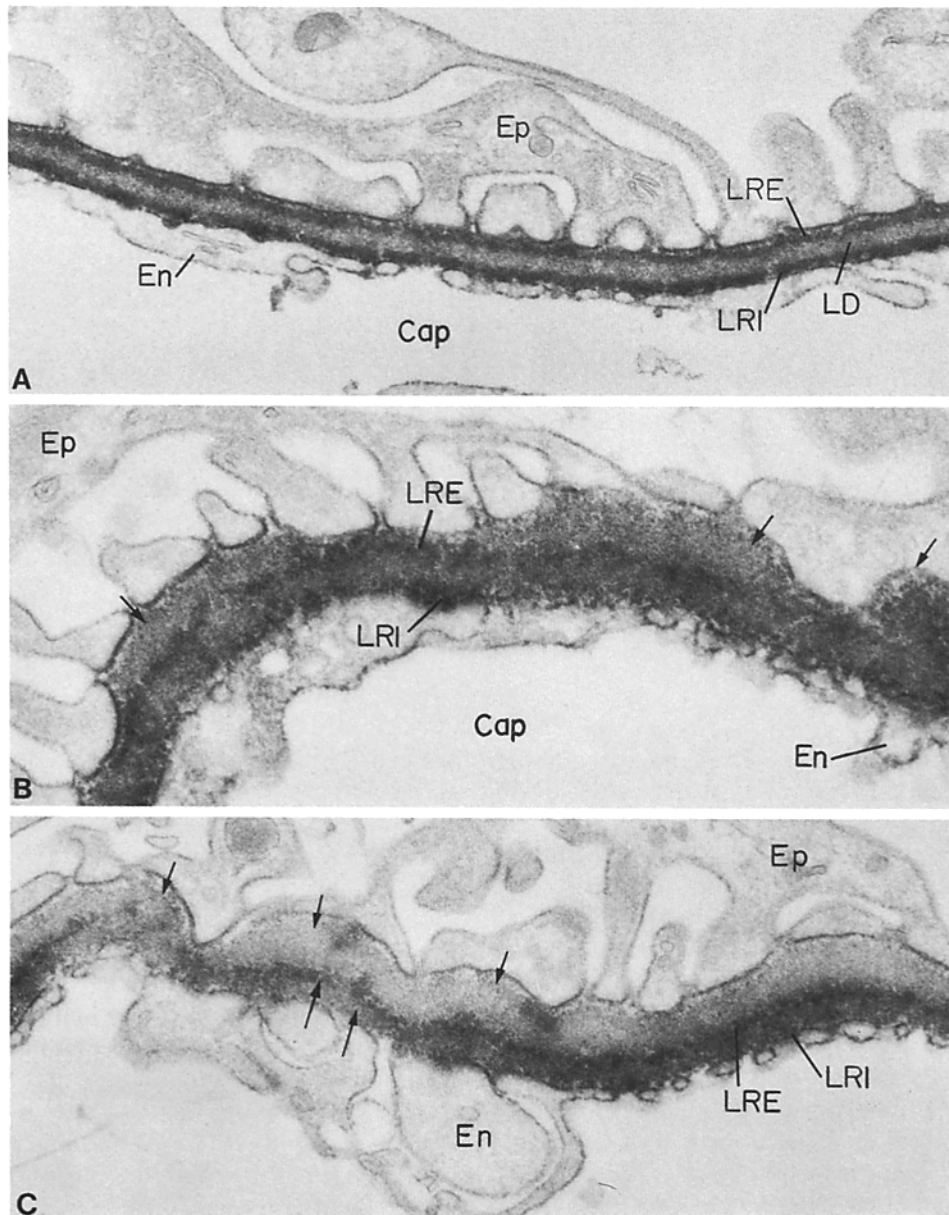


FIGURE 8. Preparations similar to those in Fig. 7. (A) Shows widespread, irregular thickening of the GBM in the glomerulus of a rat killed 19 d after injection of 20 mg of anti-HSPG(GBM) IgG. The thickening is marked in some portions of the capillary loops (*arrows*) and is minimal in others. (B) Higher magnification view of a region of thickened GBM. Several successive layers of basement membrane-like material can be detected in the thickened areas (*arrows*). (A)  $\times 12,000$ ; (B)  $\times 26,000$ .



**FIGURE 9.** Localization of rabbit IgG in the GBM of rats at various times after injection of 20 mg of anti-HSPG IgG. (A) 2 h postinjection. Rabbit IgG is concentrated in the lamina rara interna (LRI) and externa (LRE) of the GBM, with little or no staining of the lamina densa (LD). (B and C) 2 mo postinjection. Rabbit IgG is still present in the GBM and deposits of basement membrane-like material are seen on the epithelial side of the GBM, giving it a scalloped appearance. The new layer of basement membrane is only weakly reactive (B) or unreactive (C) for bound IgG (short arrows). In some areas, the original laminae rarae (LRI, LRE) can be detected (B), but in other areas (C) the stained LRI has disappeared, leaving only the former LRE that can be recognized because it stains for rabbit IgG (long arrows, B). (A)  $\times 26,000$ ; (B)  $\times 50,000$ ; (C)  $\times 32,000$ .

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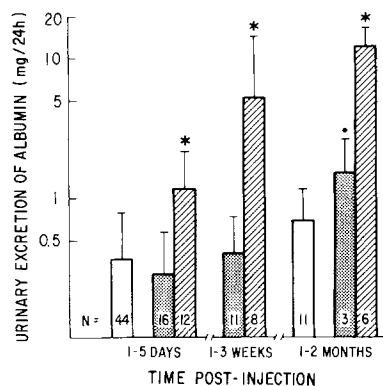


FIGURE 10. Histogram showing the dose-dependent increase in the urinary excretion of albumin caused by the binding of anti-HSPG(GBM) IgG to rat glomeruli. Stipled bars, mean albuminuria of rats injected with 3–5 mg anti-HSPG(GBM) IgG. Hatched bars, mean albuminuria of rats given 12–20 mg of anti-HSPG(GBM) IgG. Open bars, age-matched control rats. A statistically significant increase (marked by an asterisk) compared with controls is seen at all periods after injection of the higher dose ( $p < 0.001$ ), and at 1–2 mo after injection of the lower dose (marked by a dot) ( $p < 0.05$ ). *N*, the number of urine samples studied.

anti-HSPG(GBM) IgG caused an increase in the permeability of the GBM to albumin.

### Discussion

From the pathophysiologic, immunofluorescence, and electron microscopic results we can reconstruct the following sequence of events that occurs when antibodies specific for the core proteins of basement membrane HSPG ( $M_r = 130,000$ ) are injected into rats. The injected IgG binds immediately (by 3 min) to matrix elements in several organs (kidney, liver, spleen) and is rapidly depleted from the circulation. In the kidney, the antibody binds almost exclusively to laminae rarae of the GBM where the HSPG antigen is located (8). Although these HSPG are present in all renal basement membranes (8), the anti-HSPG(GBM) IgG binds only to the GBM where the structure of the endothelium with its open fenestrae (lacking diaphragms) allows direct access of the circulating IgG to the GBM. Presumably, the antibodies do not bind as readily to basement membranes of peritubular capillaries because the endothelium of these vessels has fenestrae and transendothelial channels with diaphragms (24) which undoubtedly retard transport. Also, the bulk of the antibody may be cleared from the renal circulation by binding to the GBM before reaching these capillaries. The binding of rabbit IgG along the subendothelial surface of the GBM immediately (by 3 min) activates complement, which is deposited exclusively in the LRI and attracts mononuclear and PMN leukocytes to the glomeruli. These cells adhere to the capillary wall and project pseudopods through the endothelial fenestrae to contact the immune complexes in the LRI. After 4 d, the number of attracted leukocytes decreases and C3 is no longer detectable, suggesting that these cells may be responsible for the removal or denaturation of C3. However, the injected anti-HSPG(GBM) IgG remains firmly bound to the laminae rarae and is detectable for long periods (up to 2 mo). The binding of anti-HSPG(GBM) IgG to its

antigen concentrated in the laminae rarae results in a dose-dependent, progressive thickening of the GBM which is due to the laying down of a new basement membrane layer beneath the glomerular epithelium.

The overall response to the injected anti-HSPG(GBM) IgG, i.e., binding in a linear pattern to the GBM, C3 deposition, leukocyte infiltration, and proteinuria, is typical of the acute phase of "nephrotoxic," anti-GBM nephritis (12) but is quite different from that found in Heymann nephritis, an experimental model of membranous glomerulonephritis (11). It is also similar to the response seen in rats or mice given antibodies prepared against purified basement membrane components, such as laminin (23, 25, 26), and type IV collagen (25, 26), except that proteinuria was not detected in the acute phase after injection of the latter antibodies. The proteinuria induced by anti-HSPG(GBM) IgG was clearly dose-dependent since it appeared early (during the first 5 d) only in those animals given 12–20 mg, and it increased with time but was still modest after 2 mo compared with that seen in classical anti-GBM nephritis (27). In fact, the pathologic and pathophysiologic consequences of injecting anti-HSPG(GBM) IgG were less severe than those induced by anti-GBM antibodies. The milder nature of the response cannot be explained on the basis of the amount of IgG given, because the amount of anti-HSPG(GBM) IgG we estimate to be bound after injection of 20 mg (72  $\mu\text{g/g}$  kidney) is similar to the amount of bound anti-GBM IgG (75  $\mu\text{g/g}$ ) required to induce severe proteinuria (27). The milder nature of the response may be explained by the fact that one is dealing with a monospecific antibody rather than an antibody mixture. The traditional nephrotoxic antibodies have been prepared using whole cortical homogenates, whole glomeruli, or crude GBM preparations as immunogens, and undoubtedly recognize multiple glomerular antigens.

It should be mentioned that patients with anti-GBM glomerulonephritis have circulating antibodies against noncollagenous and collagenous components of the GBM (28–30), and autoantibodies against the nonhelical carboxyterminal domain (NC-1) of type IV collagen have been implicated as the causative factor (31, 32) in Goodpasture's syndrome, a rapidly progressive form of the disease. So far, however, it has not been possible to produce an equally severe disease in experimental animals given antibodies against type IV collagen (25, 26). It can be concluded that, in all likelihood, factors such as species differences and the specificity and amount of anti-GBM antibodies given may affect the renal response.

Several of the present findings are novel or noteworthy: (a) This is the first situation in which antibodies raised against a basement membrane component were found to bind exclusively to the laminae rarae of the GBM. In anti-GBM disease (33–35), or after injection of anti-type IV collagen (36) or anti-laminin IgG (23, 36), or antibodies raised against another ( $M_r = 400,000$ ) population of HSPG isolated from the EHS sarcoma (36), the antibodies bound to all three layers of the GBM; (b) we showed that C3 bound only to the LRI, and to our knowledge, this is the first example of selective binding of C3 to the LRI. C3 has not been localized previously at the EM level in anti-GBM disease, and in immune complex disease (induced with BSA), it was found in all layers of the GBM (37); and (c) the subepithelial thickening of the GBM that results from



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anti-HSPG(GBM) IgG binding is novel. Injection of anti-type IV collagen or anti-laminin IgG also led to GBM thickening, but the patterns were different as the binding led to a uniform thickening of the GBM (25) in the former or to subendothelial thickening in the case of the latter (23). Subepithelial GBM thickening of the type induced by bound anti-HSPG(GBM) IgG has been described in some types of glomerular nephropathy (e.g., Alport's syndrome) in humans (38).

The induction of subepithelial GBM thickening suggests that the binding of anti-HSPG(GBM) IgG disturbs, in ways that are not yet understood, the turnover of the GBM. The available evidence based on silver nitrate labeling (39) indicates that basement membrane constituents are added to the GBM along the epithelial surface and removed along the endothelial-mesangial surface. Normally, synthesis and removal remain balanced so that a fairly uniform thickness is maintained. The turnover of the GBM as a whole (39), as well as of collagenous components of the GBM (40), is very slow (6–12 mo), whereas the turnover of the sulfated glycosaminoglycan side chains of proteoglycans isolated from whole kidney cortex (41) or the GBM (42) is much more rapid (3–5 d). We have previously obtained autoradiographic (2) and immunocytochemical (8) evidence indicating that, among glomerular cell types, the epithelium is the main producer of basement membrane HSPG. Apparently the binding of antibodies to these HSPG perturbs the deposition and/or removal of GBM components and leads to the accumulation of a new and thicker GBM layer. Whether this is due to increased biosynthesis of GBM components by the epithelium or decreased degradation (removal) of old basement membrane cannot be determined from the information at hand. Biosynthetic studies on the synthesis and turnover of HSPG and other GBM components after radiolabeling are required to define more precisely the nature of the metabolic defects involved.

The anti-HSPG(GBM) IgG remained bound to the laminae rarae and demonstrable by immunoperoxidase staining up to 2 mo postinjection. The bound immune complexes did not form the large, electron-dense aggregates characteristic of membranous nephritis. This resembles the situation seen after injection of anti-laminin antibodies (23), but it is quite different from that seen in Heymann nephritis (43–45) or after implantation of nonrenal antigens such as cationized proteins (46). The difference can undoubtedly be explained by the fact that immune complexes must undergo condensation and aggregation to form large electron-dense deposits (47, 48), and those formed from implanted cationized antigens (46) or gp330 (the Heymann nephritis antigen) (14) are capable of such rearrangements (45), whereas complexes formed with GBM antigens, such as basement membrane HSPG, are not, presumably because they remain firmly fixed to the GBM.

These observations help us understand the pathogenesis of human glomerular nephropathy because they show that the consequences of antibody binding to the GBM, i.e., whether resulting in an anti-GBM (12) or immune complex (11) type of nephropathy, do not depend on the original location of the antigen (LRI, LRE, lamina densa, or epithelial cell surface) or on whether it is exogenous or endogenous. The outcome depends instead on whether the antigen is firmly

fixed or free, allowing for molecular rearrangement (45, 47, 48) of the resultant immune complexes after binding antibody.

Another aspect of our findings which may be instructive is the demonstration that the transient presence of circulating antibodies that recognize a single basement membrane constituent can, in a clinically rather nonflamboyant, silent manner, initiate GBM thickening, a change commonly seen in human nephropathy. Furthermore, the recent finding of anti-HSPG antibodies in sera of human patients (6) raises the intriguing possibility that circulating anti-HSPG autoantibodies could be the direct causative agent responsible for glomerular disease in some patients.

### Summary

Antibodies specific for the core protein of basement membrane HSPG ( $M_r = 130,000$ ) were administered to rats by intravenous injection, and the pathologic consequences on the kidney were determined at 3 min to 2 mo postinjection. Controls were given antibodies against gp330 (the pathogenic antigen of Heymann nephritis) or normal rabbit IgG.

The injected anti-HSPG(GBM) IgG disappeared rapidly (by 1 d) from the circulation. The urinary excretion of albumin increased in a dose-dependent manner during the first 4 d, was increased 10-fold at 1–2 mo, but remained moderate (mean = 12 mg/24 h). By immunofluorescence the anti-HSPG(GBM) was seen to bind rapidly (by 3 min) to all glomerular capillaries, and by immunoperoxidase staining the anti-HSPG was seen to bind exclusively to the laminae rarae of the GBM where it remained during the entire 2-mo observation period. C3 was detected in glomeruli immediately after the injection (3 min), where it bound exclusively to the lamina rara interna; the amount of C3 bound increased up to 2 h but decreased rapidly thereafter, and was not detectable after 4 d. Mononuclear and PMN leukocytes accumulated in glomerular capillaries, adhered to the capillary wall, and extended pseudopodia through the endothelial fenestrae to contact in the LRI of the GBM where the immune deposits and C3 were located. At 1 wk postinjection, staining for C3 reappeared in the glomeruli of some of the rats, and by this time most of the rats, including controls injected with normal rabbit IgG, had circulating anti-rabbit IgG (by ELISA) and linear deposits of rat IgG along the GBM (by immunofluorescence). Beginning at 9 d, there was progressive subepithelial thickening of the GBM which in some places was two to three times its normal width. This thickening was due to the laying down of a new layer of basement membrane-like material on the epithelial side of the GBM, which gradually displaced the old basement membrane layers toward the endothelium.

The results show that the core proteins of this population of basement membrane HSPG ( $M_r = 130,000$ ), which are ubiquitous components of basement membranes, are exposed to the circulation and can bind anti-HSPG(GBM) IgG in the laminae rarae of the GBM. Binding of these antibodies to the GBM leads to changes (C3 deposition, leukocyte adherence, moderate proteinuria, GBM thickening) considered typical of the acute phase of anti-GBM glomerulonephritis. Antibody binding interferes with the normal turnover of the GBM, presu-

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ably by affecting the biosynthesis and/or degradation of basement membrane components.

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