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Permeation of Endogenous IgG with an Anionic Subpopulation into Glomerular Basement Membrane in Rat[1] (42436)

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Abstract. Conflicting results of previous electron microscopy studies and concerns about the validity of immunoperoxidase technique employed in those studies to accurately localize endogenous IgG in rat glomerular basement membrane (GBM) prompted us to use other techniques to answer the following question: Does endogenous IgG permeate the matrix of GBM? Immunofluorescence, radioimmunoassay (RIA), isoelectric focusing, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunodetection on Western blots were used to detect endogenous IgG in GBM. Direct immunofluorescence of normal frozen rat kidney sections prepared from *in vivo* perfused kidney showed endogenous IgG in a linear pattern of staining in the GBM. RIA for rat IgG found the IgG content of collagenase-solubilized GBM to be 0.48% of the dry weight. Immunodetection for rat IgG on Western blots of SDS–PAGE-separated GBM demonstrated endogenous IgG in purified collagenase-solubilized GBM. IgG was detected as an intact molecule with covalently linked light and heavy chains and not as small immunoreactive catabolic fragments. Isoelectric focusing followed by immunodetection on Western blot showed that part of the endogenous IgG in GBM was anionic. The results clearly show that under normal conditions, endogenous IgG can permeate into the collagen matrix of GBM in rat and that some of this IgG is more anionic than the IgG in serum. These findings may assist in understanding the transit of autoantibodies to subepithelial glomerular antigens located beneath the matrix of GBM in membranous glomerulonephropathy.

Glomerular permeability to endogenous IgG in rat has been studied earlier morphologically at the ultrastructural level by immunoperoxidase techniques (1–5). Conflicting results have emerged from these studies, and it is not clear whether endogenous IgG remains in the lumen of the capillary or has the ability to penetrate the entire matrix of glomerular basement membrane (GBM) (1–5). Evidence for both of these possibilities has been presented in the above studies (1–5). Concerns, however, have been raised recently regarding the reliability of the immunoperoxidase technique in accurately localizing the site of reaction by Courtoy *et al.* (6). Using an ingenious technique of double labeling (implantation of ferritin in GBM followed by its detection with peroxidase-labeled antiferritin antibody), these authors have convincingly shown that the peroxidase reaction product can diffuse over long distances and bind nonspecifically to unrelated structures, thereby producing false positive results. In view of these findings the question whether the endogenous IgG can penetrate the GBM needs to be reexamined. We attempted to answer this question by utilizing other sensitive techniques to detect endogenous IgG in GBM. The question of the penetration of endogenous IgG in GBM is important because it may assist in understanding the mechanism of the *in situ* immune complex formation, particularly in the case of subepithelial immune complexes. We used the techniques of immunofluorescence, radioimmunoassay, isoelectric focusing, SDS–PAGE, and immunodetection in Western blots to detect permeation of endogenous IgG into the GBM.

Materials and Methods. *Materials.* Male Lewis rats, weighing 150–200 g, were obtained from Charles River Breeding Laboratory, Inc. (Wilmington, Mass.). Tissue Tek OCT compound was obtained from Miles Laboratories, Inc. (Naperville, Ill.). Fluorescein isothiocya-

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nate (FITC)-labeled $F(ab)_2$ portion of goat anti-rat IgG (α chain specific), FITC-labeled goat anti-rabbit IgG, FITC-labeled goat anti-human IgG, and peroxidase-labeled goat anti-rat IgG were obtained from Cappel Laboratory (Cochranville, Pa.). Protein A-rich *Staphylococcus aureus* cells (PANSORBIN) were obtained from Calbiochem–Behring (San Diego, Calif.). Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) grade chemicals for SDS–PAGE were obtained from Bio-Rad Laboratories, (Richmond, Calif.). $Na^{125}I$ was obtained from Amersham Corporation (Arlington Heights, Ill.). Nitrocellulose membranes for Western blots were obtained from Millipore Corporation (Bedford, Mass.). Collagenase (from *Clostridium histolyticem* type VII) was obtained from Sigma Chemical Company (St. Louis, Mo.). Stained standard markers for SDS–PAGE and Western blots were obtained from Bethesda Research Laboratory (Gaithersberg, Md.).

Immunofluorescence method. Normal Lewis rats were anesthetized with ether and the left kidney was perfused with phosphate-buffered saline (PBS), pH 7.2, at a pressure of 110 mm Hg as previously described (7). Tissue pieces from each pole of the kidney were embedded in Tissue Tek OCT compound and snap frozen in isopentane precooled (−160°C) in liquid nitrogen. Sections (2- to 4-μm thick) were cut in a cryostat. The sections were air-dried at room temperature for $\frac{1}{2}$ hr and then either used for immunofluorescence immediately or stored at −70°C for later use. The sections were washed three times (20 min each time) in PBS, pH 7.2, on a shaker and then reacted with FITC 1:20 dilution of labeled $F(ab)_2$ portion of anti-rat IgG for 30 min in an air-tight moist chamber. The sections were washed again as above, mounted in solution of 1:9 glycerol and PBS, and examined under epillumination with a Zeiss Universal II research microscope. Controls included sections stained with unrelated FITC-labeled IgG (anti-rabbit IgG), FITC-labeled anti-rat IgG absorbed with rat IgG, a positive rat serum containing anti-GBM autoantibodies, and sections reacted with rabbit anti-rat IgG (α chain specific) serum followed by staining with either FITC-labeled anti-rat IgG (blocked sections) or FITC-labeled anti-rabbit IgG (enhanced staining).

Radioimmunoassay (RIA) for rat IgG. Purified rat IgG was labeled with ^{125}I by the chloramine T method to a specific activity of 18 μCi/μg. Eighty-nine percent of the label could be precipitated by trichloracetic acid (TCA). RIA was carried out in 12 × 75-mm glass tubes and RIA buffer (PBS, pH 7.4, in 0.2% bovine serum albumin) was used to dilute all reagents. Antibody to rat IgG, rabbit anti-rat IgG (RIA grade), was titrated using a constant amount of ^{125}I-labeled rat IgG (20,000 counts per minute (cpm)) and a varying amount of the antibody diluted in RIA buffer in 10-fold serial dilutions. The antibody dilution in which limiting amount of antibody would be available was found to be 1:10,000 (1:30,000 in the final incubation mixture). This dilution of the antibody was then used in the competitive inhibition assay for rat IgG as follows: 50 μl of standard rat IgG (0.01–10 mg/ml) or samples of collagenase-solubilized GBM, 50 μl of ^{125}IgG containing 20,000 cpm, and 50 μl of 1:10,000 diluted anti-rat IgG were placed into glass tubes. Controls of total counts without antibody and rat IgG, and only antibody without antigen (maximum binding), were included and all samples were run in duplicates. The tubes were vortexed and incubated overnight at 4°C. The next morning 20 μl of 10% suspension of protein A-rich *staphylococcus aureus* cells (PANSORBIN) was added, and the tubes were vortexed and incubated for 30 min at room temperature. RIA buffer (1 ml) was then added to all tubes, and the tubes were vortexed and centrifuged at 2000*g* for 10 min at 4°C. The sediment consisted of firm pellets. The supernatant was decanted, the inside of the tubes carefully wiped dry with filter paper without disturbing the pellets, and the sediment counted for radioactivity in a gamma counter (Beckman) for 1 min. Counts were corrected for controls (background activity and nonspecific binding to PANSORBIN) and the mean was determined from duplicates. A standard inhibition curve was drawn with the range 0.01 to 10 mg/ml of IgG and the values for the samples were determined from the standard curve.

IgG in GBM. Collagenase-solubilized GBM was prepared from *in vivo* perfused bloodless normal rat kidneys according to the previously described methods (8, 9). Briefly, Lewis rats (n = 30) weighing 200–300 g were anesthetized

by ether and their kidneys perfused *in vivo* with PBS, pH 7.2. The cortex was separated from medulla and chopped into a thick paste with scissors and a razor blade. The glomeruli were isolated by the sieving technique. Renal cortical tissue from 50 kidneys was passed through size 60 (250-μm opening) and size 150 (150-μm opening) stainless steel mesh screens placed in series and collected on size 100 mesh screen (75-μm opening) by rinsing with large volumes of cold (4°C) PBS. Stainless steel mesh sieves were obtained from Arthur H. Thomas and Company (Philadelphia, Pa.). Over 90–95% of glomeruli obtained were free of Bowman's capsule with minimal tubular contamination. The isolated glomeruli were washed three times with PBS by centrifugation in a conical centrifuge tube at 400*g* at 4°C. The washed glomerular pellet was suspended in 1 *M* NaCl and sonicated. The sonicated material was washed six times with cold 1 *M* NaCl by centrifugation at 4°C at 3000*g* for 10 min each time. The pellet was then washed with 4°C deionized water by centrifugation as described above and lyophilized. Fifty milligrams of lyophilized GBM was suspended in 0.02 *M* Tris–MCl buffer, pH 7.3, containing 0.005 *M* $CaCl_2$ and 0.01% sodium azide and reacted with 500*g* of collagenase for 24 hr at 37°C on a shaker. It was then centrifuged at 4°C for 30 min at 15,000*g*. The supernatant was removed and dialyzed extensively against deionized water, lyophilized, and stored at −70°C. Lyophilized glomeruli and collagenase-solubilized GBM were dissolved in RIA buffer to a concentration of 200 μg/ml, and their IgG content was determined by RIA.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting. IgG, standard stained markers, and collagenase-solubilized normal rat GBM were separated by SDS–PAGE in 8% polyacrylamide slab gels under nonreducing and reducing conditions (5% mercapoethanol) according to the discontinuous buffer system of Laemmli (10). Electrophoresed peptides were transblotted to nitrocellulose according to the method of Towbin *et al.* (11) using 25 m*M* Tris, 192 m*M* glycine, and 20% methanol as the transfer buffer. Transblotting was carried overnight at 0.15 A. The leftover polyacrylamide slab gel was stained with Commassie blue (0.1% in 40% methanol, 10% acetic acid) for 1 hr and destained in 12% ethanol, 7% acetic acid. The lane with stained, separated markers and another lane with GBM peptides were cut out from the transblot. The lane containing GBM peptides was stained with 0.1% Amido black in 45% methanol, 10% acetic acid. The remainder of the transblot was blocked for 2 hr at 37°C with blocking buffer containing 5% BSA, 0.05% Tween 20 in PBS. Individual lanes from the blocked blot were then reacted for 1 hr at room temperature with rabbit anti-rat IgG 1:1000 dilution (Miles Laboratories). The blots were then washed three times, 10 min each, in PBS, 0.05% Tween 20. The blots were then reacted at room temperature for 1 hr with 1:4000 dilution of peroxidase-labeled goat anti-rabbit IgG (Capel Laboratory). Controls included reaction of strips with peroxidase-labeled goat anti-rabbit IgG only. All antisera were diluted in 3% BSA, 0.05% Tween 20 in PBS. The strips were then washed six times for 10 min each with PBS containing 0.05% Tween 20. The strips were washed again three times for 10 min each in PBS without Tween 20. The color reaction was developed by using 0.05% 4-chloro-1-napthol in 15% methanol, 0.015% H_2O_2 in PBS.

Isoelectric focusing and detection on Western blots. Isoelectric focusing was performed in 0.75-mm thick, 180 × 210-mm, 0.9% agarose gel (Igogel–FMC Corp., Rockland, Maine) in a horizontal slab gel unit (Hoefer Scientific Instruments). Carrier ampholytes in the pH range of 3–10 were obtained from Isolab (Akron, Ohio) and standard pI markers ranging from 3.6 to 10.2 were obtained from FMC Corporation. Samples of pI markers (1 μl), normal rat IgG (3 μl; 1 mg/ml) and collagenase solubilized GBM (25 μl; 3.3 mg/ml) were applied and focusing was carried out for 20 min at 2 W followed by 2 hr at 5 W. The gel was fixed and stained with silver stain using the Silver Stain Kit (Isolab). For transfer of proteins to nitrocellulose (Schleicher & Schuell, BA-85) nitrocellulose was prewet and laid on the gel. Five sheets of filter paper were placed on top of the nitrocellulose sheet and covered with a glass plate and lead weights. Transfer was allowed to continue for 4 hr. Nitrocellulose was then blocked overnight with PBS, 0.1% Tween 20. The blocked blot was incubated in 1:50 dilution of rabbit anti-rat IgG (Miles Laboratories) in PBS, 0.1% Tween,

3% BSA for 1 hr at room temperature. The blot was then washed with PBS, 0.1% Tween three times for 10 min each. For detection with enzyme-labeled second antibody the blot was reacted with 1:1000 dilution of peroxidase-labeled goat anti-rabbit IgG (Cappel Laboratory) in 3% BSA, PBS 0.1% Tween buffer for 1 hr at room temperature. It was then washed with PBS, 0.1% Tween for 10 min twice and once with PBS alone for 10 min at room temperature. The color reaction was developed by using 0.05% 4-chloro-1-napthol, in 15% methanol, 0.015% H_2O_2 in PBS. For autoradiography the blot was reacted with 5 ml (2×10^5 cpm/ml) of ^{125}I-labeled protein A for 1 hr at room temperature. The blot was washed with PBS, 0.1% Tween for 30 min five times and then exposed to X-AR5 film (Kodak) at −70°C with intensifying screen. Controls included reaction with nonimmune first antibody serum (normal rabbit serum) and with the labels alone (^{125}I-labeled protein A and peroxidase-labeled goat anti-rabbit IgG). Protein A (Pharmacia) was labeled with ^{125}I using the chloramine T method.

Results. *Immunofluorescence.* Normal kidney sections obtained from perfused kidneys (n = 15) showed linear staining for rat IgG along the GBM (Fig. 1). The intensity of staining varied in individual glomeruli; however, it appeared to be present in all glomeruli. The staining was more intense in the part of the GBM that is adjacent to the mesangium; however, it was clearly seen in the part forming the capillary loops. The staining was similar to that usually seen with anti-GBM antibody and would be difficult to differentiate from the latter (Fig. 2). The staining was specific for rat IgG as demonstrated by the control experiments described below. No staining was seen when the sections were stained with FITC-labeled anti-rabbit IgG, FITC-labeled anti-human IgG, or with FITC-labeled anti-rat IgG earlier absorbed with rat IgG (Fig. 3). Also the staining could be blocked if the sections were first reacted with unconjugated rabbit anti-rat IgG serum (blocked sections) and then stained with FITC-labeled rabbit anti-rat IgG. Furthermore, the staining was enhanced if the same blocked sections were stained with FITC-labeled anti-rabbit IgG. Sections obtained from unperfused kidneys that had been quickly removed from an anesthesized rat without clamping renal vessels showed an even greater amount of IgG staining in the glomerulus, and in addition, showed staining in the peritubular capillaries (Fig. 4).

RIA for IgG. The IgG content of collagenase-solubilized GBM obtained from three different preparations of 50–60 kidneys each

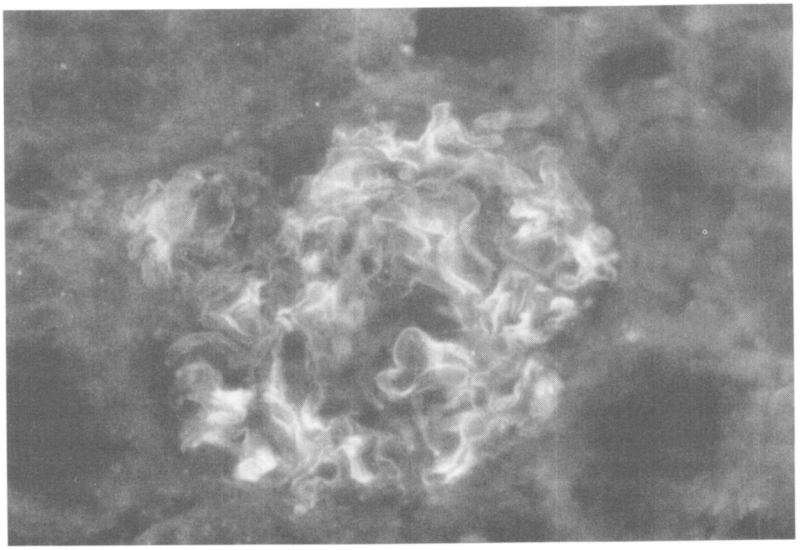

FIG. 1. Normal rat kidney frozen section obtained from *in vivo* perfused kidney and stained with FITC-labeled $F(ab)_2$ portion of anti-rat IgG. Linear staining along the GBM is seen (magnification 400×).

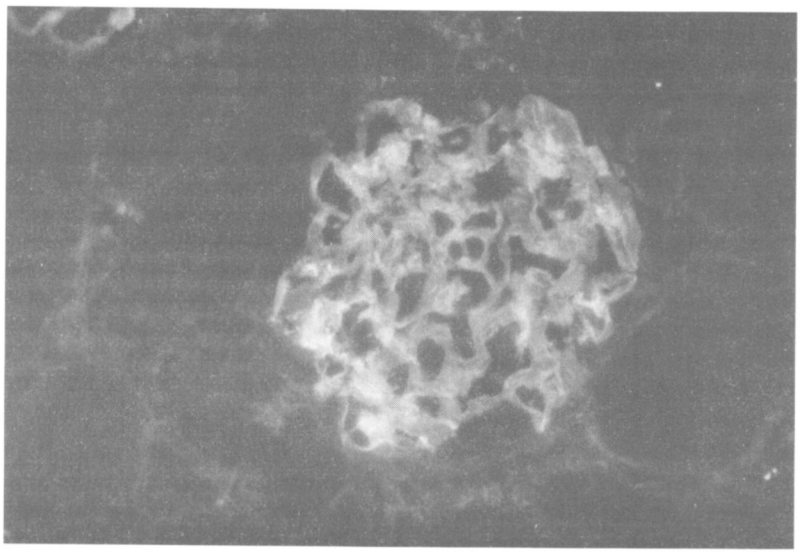

FIG. 2. Kidney from a Brown Norway rat injected with mercuric chloride to induce anti-GBM autoantibody. Unfixed frozen section stained with FITC-labeled $F(ab)_2$ portion of anti-rat IgG. Linear staining is seen along the GBM (magnification 400×).

ranged from 0.435 to 0.550% (mean 0.48) of the dry weight. The IgG content of whole glomeruli from one preparation of 50 kidneys was 0.017%.

pI of IgG and its detection on Western blots. The IgG in collagenase-solubilized GBM was more anionic than the serum IgG. The pI of serum IgG ranged from 7.0 to 8.6 and the pI

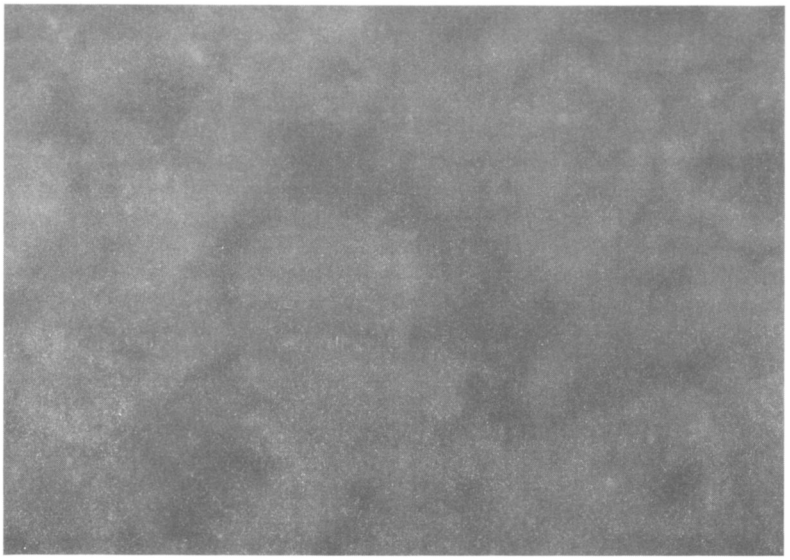

FIG. 3. Normal rat kidney frozen, unfixed section obtained from *in vivo* perfused kidney and stained with FITC-labeled $F(ab)_2$ portion of anti-rat IgG which had been absorbed with normal rat IgG. No staining is seen in the glomerulus (magnification 400×).

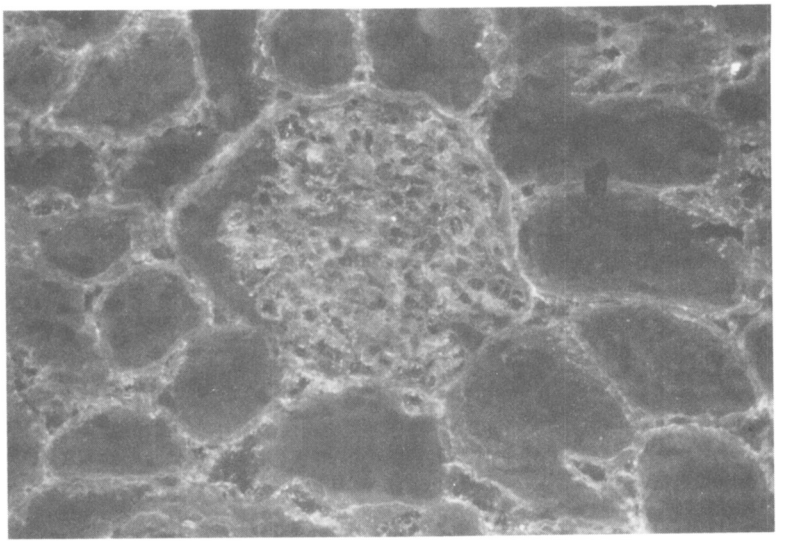

FIG. 4. Normal rat kidney frozen unfixed section from unperfused kidney stained with FITC-labeled $F(ab)_2$ portion of anti-rat IgG. In addition to staining of the glomerulus, peritubular capillaries are also stained (magnification 250×).

of IgG in GBM from 6 to 7.7 (Fig. 5). Clearly there was a population of IgG in the GBM IgG which was anionic and which could not be detected in the serum IgG.

IgG in Western blots of GBM. Two distinct polypeptide bands corresponding to the heavy and light chains of IgG were seen when collagenase-solubilized GBM was separated in SDS–PAGE under reducing conditions with 5% mercaptoethanol. Without reduction a single band corresponding approximately to the IgG molecular mass of 180,000 Da was seen (Fig. 6).

Discussion. Our results clearly show that endogenous IgG, included in which is a distinct anionic subpopulation, is present in GBM and that the entire molecule can penetrate at least that part of GBM which can be solubilized by collagenase. The IgG detected on Western blots contained only the light and heavy chains when reducing conditions were used, and a single band when no reduction with mercaptoethanol was used. These results show that the intact molecule and not the various catabolic fragments of IgG that might have been detected immunologically penetrated the collagen matrix of the GBM. The inherent superiority of the technique of Western blotting in this respect is an advantage over the immunoperoxidase technique. In the lat-

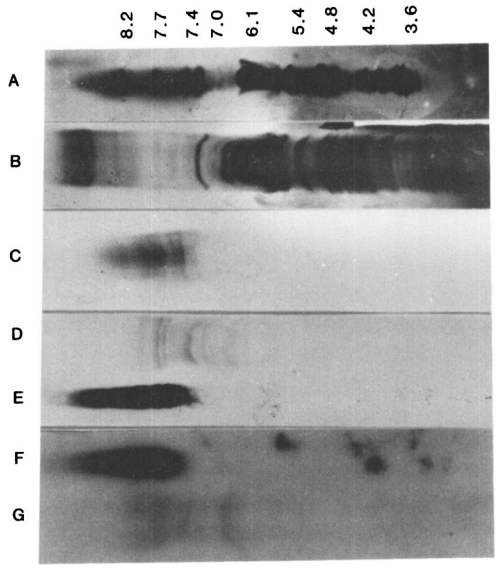

FIG. 5. Isoelectric focusing of IgG in GBM and normal IgG and detection on Western blots. Lane A, pI markers—silver stained; lane B, collagenase-solubilized GBM proteins—silver stained; lane C, normal serum IgG—silver stained; lane D, IgG in collagenase-solubilized GBM detected by indirect immunoperoxidase staining; lane E, normal serum IgG detected by indirect immunoperoxidase staining; lane F, normal serum IgG detected by indirect immunoprotein A autoradiography; lane G, IgG in collagenase-solubilized GBM detected by indirect immunoprotein A autoradiography.

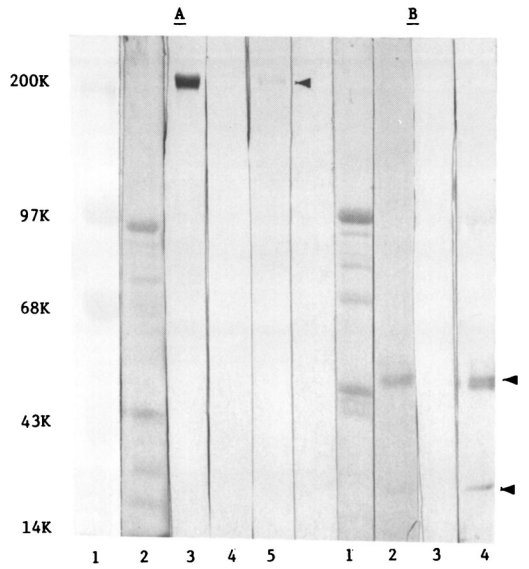

FIG. 6. Western blot of collagenase-solubilized GBM and normal serum IgG. Lane A.1, standard molecular weight markers; lanes A2–5 are unreduced and lanes B1–4 are reduced; lanes A2, 4, 5 and B1, 3 and 4 are collagenase-solubilized GBM; lanes A3 and B2 are normal serum IgG. Lanes A2 and B1 are amido black stain. Lanes A3 and 5 and B2 and 4 are first reacted with rabbit anti-rat IgG and then with peroxidase-labeled goat anti-rabbit IgG. Lanes A4 and B3 are reacted with peroxidase-labeled goat anti-rabbit IgG only. Arrow on A5 indicates intact IgG. Arrows on B4 indicate heavy and light chains of IgG.

ter, peroxidase-labeled anti-rat IgG will detect the intact light and heavy chains or their fragments if the antibody is not heavy chain specific, and detect only heavy chain or its antigenic fragments if it is specific for heavy chain. The technique cannot tell whether the detected reaction in the GBM is due to a smaller antigenic fragment (that might get filtered) or due to the intact IgG molecule. Even if light and heavy chain specific reagents conjugated with different fluorescent labels are used separately on the same sample and both heavy and light chains are detected, it is not possible to know whether the light and heavy chains are covalently attached and present as intact IgG molecules or present as separate complete or incomplete heavy and light chain antigenic fragments.

All previous studies have used the immunoperoxidase technique and electron microscopy to study the penetration of rat GBM by the endogenous IgG (1–5). Using peroxidase-labeled anti-rat IgG to localize endogenous rat IgG, Ryan *et al.* (1) found that when superficial glomeruli in anesthesized Munich–Wistar rats were rapidly fixed *in situ* by dripping glutaraldehyde onto the renal surface, IgG was largely confined to the glomerular capillary lumen, with only small amounts in the lamina rara interna immediately beneath the endothelial fenestrae, and none deeper in the GBM or in the urinary space. When cortical tissue was subjected to routine immersion fixation, or when the fixation was performed *in situ* after ligation of the renal artery, IgG was found throughout the GBM. Similar results were found for rat albumin. They proposed that under normal conditions endogenous IgG *does not* penetrate significantly beyond the endothelial layer of GBM and stressed the importance of good blood flow conditions. Laliberte *et al.* (2, 3), however, disagreed with these findings and found no differences in the penetration of endogenous IgG between the *in situ* fixed and the immersion-fixed tissues. In both cases albumin could be easily localized in the urinary space and the IgG in the lamina rara interna and lamina densa. They felt that *in situ* fixation did not necessarily represent good blood flow conditions and immersion fixation as poor flow conditions. They considered the immersion fixation technique to be adequate for the study of endogenous proteins and cited the detection of albumin in the glomerular filtrate by micropuncture studies to support their contention (12–14). Our studies were performed on kidneys that had been perfused *in vivo* or on those that were removed quickly without clamping renal vessels. The latter tissue would be comparable to the immersion fixation tissue. In both situations we found IgG in the GBM.

Laliberte *et al.* (3) also conducted their studies with an additional superior technique. They immunized rats with horseradish peroxidase (HRP) to induce anti-peroxidase IgG antibody and then detected the localization of antibody (endogenous IgG) in GBM by using HRP (molecular weight 40,000) as the probe. This technique overcomes the difficulty of inconsistent penetration of HRP-labeled antibody (larger molecule). They concluded that endogenous IgG penetrated the GBM but did not cross it. Olivetti *et al.* (4) in a later study

using the same techniques found that while the albumin penetrated the GBM and appeared in the urinary space the endogenous IgG was mainly confined to the capillary lumen and did not significantly penetrate beyond the subendothelial fenestrae. Since a consensus does not emerge from the above studies and since concerns regarding the reliability of the immunoperoxidase technique have been raised recently by Courtoy *et al.* (6), it is difficult to draw firm conclusions from these studies as to the penetration of the endogenous IgG into GBM in rat.

Courtoy *et al.* (6) implanted an electron dense tracer, cationic ferritin, in laminae rarae interna and externa of GBM, and subsequently localized the ferritin by HRP-labeled antiferritin IgG. The coincidence between the location of the reaction product and the ferritin clusters was assessed. They found that the oxidized substrate (diaminobenzidine) reaction product could diffuse over long distances beyond the site of the specific reaction site and adsorb nonspecifically to other unrelated structures. Even under the limited incubation conditions some diffusion was encountered. In view of these findings, the results of earlier studies as to the location of endogenous IgG in GBM are difficult to interpret, and the question whether the endogenous IgG penetrates the GBM is difficult to answer.

Some of the IgG in GBM is a subpopulation of IgG molecules which are anionic. These results suggest some form of selective specific binding since this anionic moiety was not seen in normal serum IgG. The latter most likely contains this anionic moiety in low concentration which probably accounted for its undetectability in the methods used by us. What is the mechanism of this selective accumulation? One possibility is that this is the result of electrostatic binding to some cationic molecules in GBM. This possibility was considered by Melvin *et al.* (15) to explain their finding of IgG_4 in human GBM. These investigators used subclass specific antisera to detect IgG_4 by indirect immunofluorescence in normal human kidney sections. They noted that while they found IgG_4 they did not detect IgG_1, IgG_2, and IgG_3 despite the fact that the latter subclasses were the predominant forms of IgG in normal serum. They interpreted this to mean that IgG_4 was selectively bound by GBM and not simply trapped from blood. Although they did not isolate IgG from GBM and determine its pI they cited previous work where it had been shown that the pI of normal human IgG_4 ranged from 5.8 to 6.0. Based on this they suggested that IgG_4 was bound to some cationic proteins in GBM through electrostatic forces. Our findings in rat agree with their results in humans although different experimental approaches were used in the two studies. In rat the IgG classes are different (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_{2c}) with IgG_{2a} and IgG_{2b} being the predominant subclasses in serum. The pI of rat IgG subclasses have not been determined previously. Our results in this study show that most of the serum rat IgG has a pI range of 7 to 8.6. Which of the known subclasses in rat corresponds to human IgG_4 is not known. Our results clearly show that an anionic subpopulation is selected by the collagenase soluble part of GBM. These findings, as pointed out by Melvin *et al.* (15), are in clear opposition to the current theory that only cationic macromolecules cross easily the anionic GBM, the anionic ones being repulsed. However, Izui *et al.* (16) have demonstrated that anionic DNA can bind to GBM *in vitro,* a finding consistent with our and the Melvin *et al.* (15) results. Further studies will be required to detect cationic proteins in GBM to clarify the issue.

Another possibility for binding of IgG to GBM is that the IgG bound is more heavily glycosylated than the IgG which does not bind. It has recently been shown that nonenzymatic glycosylation of ferritin, even when the latter is anionic, increases its transglomerular transport (17). Additionally, Brownlee *et al.* (18) have shown that nonenzymatically glycosylated GBM collagen *in vitro* can increase the binding of plasma proteins, including IgG. Since we studied normal rat GBM it is unlikely that the latter was a possible mechanism; however, the former mechanism remains a possibility.

Westberg and Michael (19) previously detected *human* IgG in normal human GBM. Their studies were performed on GBM prepared from unperfused kidneys obtained at autopsy and a relatively insensitive technique of radial immunodiffusion (Ouchterlony) was used to detect IgG. In addition to IgG they also found other plasma proteins (albumin,

C3 complement, fibrinogen), including the heavy molecular weight protein IgM. Since unperfused kidneys were used and since many plasma proteins including a large protein (IgM) were detected with a relatively insensitive technique, it is likely that their preparation of GBM was contaminated with blood proteins. Therefore, it is difficult to know whether the detected IgG was present in the GBM. Recently, however, as mentioned, Melvin *et al.* (15) have convincingly shown by indirect immunofluorescence that IgG_4 can be detected in some normal human kidneys. IgG_4 was detected in a linear pattern along the GBM.

Our finding IgG in collagenase-solubilized GBM suggests that IgG was not simply a contaminant from blood because (i) the GBM was prepared from the *in vivo* perfused kidneys, (ii) there are multiple washing steps during the preparation of GBM and its subsequent solubilization by collagenase, and (iii) it was more anionic than serum IgG. However, Houser *et al.* (20) have previously shown that contamination with mesangial matrix can occur during preparation of rat GBM when the latter is prepared from *unperfused* kidneys. They found mesangial matrix antigens in sonicated GBM; however, they did not test the collagenase-solubilized fraction. Therefore, we do not know whether mesangial matrix contamination can be present in collagenase-solubilized fraction of GBM, particularly if it is obtained from *in vivo* perfused kidneys. Although we can not eliminate the possibility of mesangial matrix contamination in our preparation of GBM it is unlikely for the reasons given above that the contaminating mesangial matrix contained blood IgG unless the mesangial matrix like the GBM also selects IgG. The results of higher IgG content on weight basis in the collagenase-solubilized GBM as compared with whole glomeruli also favor the contention that IgG is intimately associated with GBM and not simply a contaminant from blood. If the latter were the case we should have seen higher amounts in whole glomeruli. Whether the IgG in GBM is an extraneous molecule of blood origin that is simply firmly fixed in the GBM matrix or it is part of the chemical structure at the molecular level is difficult to answer. However, our findings in the Western blots seem to indicate that the latter is not the case because the detected IgG was in the form of two distinct polypeptide bands that corresponded to the heavy and light chains of normal IgG and thus showed structural similarity to serum IgG. Based on the above it seems that IgG in GBM (i) is present at least in the collagenase-soluble part, (ii) is antigenically similar to normal serum IgG, (iii) is structurally similar to normal serum IgG as far as electrophoretic separation under reducing and nonreducing conditions in SDS–PAGE is concerned, (iv) contains an anionic subpopulation of IgG, (v) is present as an intact IgG molecule, (vi) is most probably not part of molecular structure of GBM, and (vii) is most likely of blood origin.

We do not know whether IgG enters the GBM in the living animal under physiologic conditions of renal perfusion or whether the IgG detected in the GBM by us and by previous investigators (1–5) is the result of interruption of renal perfusion which must occur during the removal of the kidney. Unfortunately, at present there is no methodology that can overcome this objection.

In order to understand the mechanism of *in situ* immune complex formation in GBM, particularly with glomerular antigens localized on the epithelial cell membrane, it is essential to understand the mechanism by which IgG travels from the capillary lumen into the GBM. It is well known that heterologous anti-GBM antibodies used to induce nephrotoxic nephritis readily bind to GBM following intravenous injection and when studied by immunofluorescence (21). However, the exact site of the antigenic components within the GBM that bind these antibodies has not been clearly identified (22). Recently heterologous antilaminin antibodies have been shown to bind readily to the GBM also (22, 23). However, the exact site(s) of laminin in GBM remains controversial, due in part to the difficulties of interpreting results from the immunoperoxidase technique used in these studies.

How antibodies directed against the Heymann antigen (gp600-gp330) reach their antigen site in GBM in rat or how possibly similar antibodies in human membranous glomerulonephropathy reach the antigens in the GBM of humans is not known and may be dependent on many factors. Our findings that the endogenous IgG in rat can penetrate to the

collagen matrix of the GBM may assist further studies in understanding the mechanism of transport of endogenous antibody to subepithelial site in GBM and the *in situ* complex formation.

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