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Studies with Antibodies to Cultured Rat Glomerular Epithelial Cells

Subepithelial Immune Deposit Formation After In Vivo *Injection*

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To investigate the role of glomerular epithelial cell (GEC) membrane proteins in the in situ formation of subepithelial immune deposits, the authors raised a rabbit antiserum against GEC that had been grown in culture (anti-GEC). By indirect immunofluorescence (IF) on normal rat kidney, anti-GEC stained proximal tubular brush border (BB). After intravenous injection into animals, granular glomerular capillary wall staining for IgG was present by IF and subepithelial immune deposits were identified by standard transmission and immunoelectron microscopy. Using the latter technique, injected anti-GEC IgG was identified beneath slit diaphragms and in endocytic-coated pits and intracellular vesicles of podocytes. Anti-GEC immunoprecipitated gp330 and two other proteins from radiolabeled BB. These proteins also were identified by sheep anti-rat Fx1A, the antiserum responsible for passive Heymann nephritis. Anti-GEC and anti-Fx1A also immunoprecipitated five identical proteins from surface-labeled GEC. Biosynthetically-labeled but not surface-labeled GEC contained immunoprecipitable gp330. Thus, injection into rats of antibodies raised against cultured GEC can produce subepithelial immune deposits, a disease process classically induced by antibodies to BB or its purified components. In addition to gp330, GEC and BB share other antigenic determinants that may contribute to the formation of these immune deposits. (Am J Pathol 1989, 134:1125–1133)

Passive Heymann nephritis (PHN) in rats is induced by injection of anti-Fx1A, a polyspecific antiserum raised against proximal tubular brush border (BB) antigens.[1] The injected antibodies bind to resident antigen(s) in the glomerular capillary wall, forming subepithelial immune deposits.[2,3] The predominant antigen in PHN appears to be a 330 kd glycoprotein, gp330, which is present on both BB and the glomerular visceral epithelial cell (GEC) *in vivo*.[4,5] Injection of anti-gp330 into rats leads to rapid binding of antibody to coated pits of the GEC with the subsequent formation of subepithelial immune deposits.[6]

Other BB antigens shared with GEC may be important in PHN or related disorders of *in situ* subepithelial immune complex formation.[7,8] Monoclonal antibodies reactive with 90 kd[9] and 110 kd[10] BB proteins bind to GEC in tissue sections and induce transient glomerular deposits after injection *in vivo*. A monoclonal antibody to a 107 kd BB and glomerular sialoglycoprotein binds to cultured GEC.[11] In addition, injection of this antibody into rats results in glomerular binding and the development of proteinuria if animals are simultaneously given complete Freund's adjuvant.[11] Similar results occur after injection of polyclonal antibodies to the BB protein, dipeptidyl peptidase IV (gp108).[12,13] Other relevant antigens may include a 95 kd protein present in normal glomeruli[14] and gp600, which is composed of 5 glycoproteins with M_r of 330, 140, 110, 80, and 70 kd.[15]

We and others have shown that, in addition to binding to BB and glomeruli, anti-Fx1A binds to the plasma membrane of GEC in culture.[16–20] Anti-gp330 also binds to GEC in primary culture, although the amount of binding as determined semiquantitatively by immunofluores-

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cence microscopy (IF) is less than with anti-Fx1A, suggesting that other antigens recognized by anti-Fx1A are present on GEC in culture.[17] Based on studies of GEC in culture, the current hypothesis for the formation of subepithelial immune deposits in PHN is that injected anti-Fx1A binds to the GEC plasma membrane, becomes associated with the cytoskeleton,[18] is redistributed on the cell surface, forming "caps," and subsequently is shed from the cell into the subepithelial space.[17]

In this study we show that rabbit antibodies raised against cultured GEC (anti-GEC) can induce formation of subepithelial immune deposits *in vivo*. These deposits resemble those seen in PHN produced by injection of anti-Fx1A. We also present evidence demonstrating that GEC and BB share antigenic determinants, in addition to gp330, that are recognized by anti-GEC and anti-Fx1A.

Materials and Methods

GEC Culture

GEC were cultured from Sprague-Dawley rats (CD; Charles River Breeding Laboratories, Wilmington, MA) on a collagen matrix as detailed previously.[20] GEC were characterized according to standard criteria[21,22] as described.[20] Briefly, by phase-contrast microscopy, subconfluent cells were polygonal in shape and formed a characteristic cobblestonelike monolayer on reaching confluency. By electron microscopy, junctional complexes were demonstrated. Cells were sensitive to the cytotoxic effects of the aminonucleoside of puromycin. Histochemical staining of cells for alkaline phosphatase[23] was negative, thus excluding a proximal tubular origin of the cells.[24] Cells were studied between passages 12 and 25.

Antibodies

Sheep anti-Fx1A IgG and nonimmune sheep IgG were prepared and characterized as described previously.[25] Rabbit antiserum to gp330 was a gift from Dr. John Niles (Dept. of Pathology, Massachusetts General Hospital, Boston, MA), and was raised by immunization of a rabbit with gp330 obtained by affinity-purification of rat BB with monoclonal anti-gp330.[10] The IgG fraction of anti-gp330 was isolated by protein A affinity chromatography (Pierce Chemical Co., Rockford, IL).

Anti-GEC was produced in a rabbit by immunization with GEC as follows. Confluent GEC on a collagen matrix from two 100-mm culture dishes (Falcon, Becton Dickinson and Co., Oxnard, CA) were removed and incubated in 10 ml of 0.2% collagenase (CLS IV, Cooper Biomedical, Malvern, PA) in Hank's balanced salt solution (HBSS; GIBCO, Grand Island, NY) for 60 minutes at 37 C, at which time visible collagen was digested. The cells were washed twice in HBSS, resuspended in 1 ml of HBSS, and mixed with 1 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The suspension was administered in 20 subcutaneous flank injections. After a booster immunization 1 month later, serum was collected, heat-inactivated (56 C, 30 minutes), absorbed twice with rat erythrocytes, and precipitated twice with 50% ammonium sulfate. The resulting globulin (Ig) preparation was reconstituted in half the original volume, dialyzed extensively against phosphate-buffered saline (PBS), and stored at −70 C until use. Normal rabbit Ig was similarly isolated from an unimmunized animal.

The IgG fraction of anti-GEC and normal rabbit Ig were obtained by anion-exchange chromatography, and then conjugated to horseradish peroxidase (HRP, type VI, Sigma Chemical Co., St. Louis, MO)[26] for immunoelectron microscopy (see below). Sheep anti-rabbit IgG conjugated with HRP was obtained from Cappel Laboratories (Malvern, PA).

Immunoprecipitation

BB from normal Sprague-Dawley rat kidney was obtained as described by Booth and Kenny.[27] At all steps the following protease inhibitors were present: 1.5 μM pepstatin A, 1 mM benzamidine, 0.5 mM dithiothreitol, and 1 mM diisopropyl fluorophosphate (Sigma Chemical Co.). BB was solubilized in PBS containing 1% Triton X-100 (Pierce Chemical Co.) and protease inhibitors for 15 minutes on ice, centrifuged at 50,000*g* for 15 minutes, and the supernatant containing solubilized BB proteins was radiolabeled with ^{125}I (New England Nuclear, Boston, MA) using Iodobeads (Pierce Chemical Co.) according to the manufacturer's instructions. Free ^{125}I was separated from protein-bound ^{125}I by chromatography on G-25 Sephadex (Pharmacia, Inc., Piscataway, NJ).

To surface label GEC, confluent GEC from five 100-mm culture dishes were collagenase treated as above, washed three times in PBS, and incubated for 15 minutes at 22 C with 100 μg lactoperoxidase (Sigma Chemical Co.), 3 U glucose oxidase (Sigma Chemical Co.), 500 μg dextrose, and 1 mCi ^{125}I in a total volume of 1 ml.[28] Cells were then washed five times in PBS containing 1% wt/vol bovine serum albumin and once in ice-cold PBS. At this point, viability of the cell preparation was >85%, as determined by trypan blue exclusion. GEC were then solubilized in 1 ml of PBS containing protease inhibitors and 1% Triton X-100 for 15 minutes on ice and centrifuged at 2200*g* for 10 minutes. The supernatant was then chromatographed on a G-25 Sephadex column as with solubilized BB.

GEC proteins were also biosynthetically labeled with ^{35}S-L-cysteine and ^{35}S-L-methionine (New England Nuclear). Nearly confluent GEC from one 100-mm culture dish were washed three times with PBS, incubated for 30 minutes at 37 C with cysteine- and methionine-free RPMI-1640 medium (GIBCO) containing 5% dialyzed fetal calf serum, washed twice with PBS, and incubated for 18 hours at 37 C with the above medium containing 200 μCi ^{35}S-L-cysteine and 200 μCi ^{35}S-L-methionine. The cells were then washed twice with PBS, and the cell layer and adherent collagen were incubated for 45 minutes at 37 C with 0.2% collagenase in HBSS. The resulting cell pellet was washed four times with PBS and lysed with PBS containing 2% Triton X-100 and protease inhibitors for 20 minutes on ice. Nuclei and insoluble debris were removed by centrifugation at 2200*g* for 10 minutes.

Immunoprecipitation of antigens was performed as described previously,[18] using 175 μg of rabbit anti-GEC, anti-gp330, or normal rabbit Ig or 300 μg of sheep anti-Fx1A or normal sheep IgG followed by 100 μg of affinity-purified rabbit anti-sheep IgG. Immunoprecipitated proteins were dissolved in reducing Laemmli sample buffer[29] and electrophoresed on a 4 to 12% gradient polyacrylamide gel. The M_r of immunoprecipitated material was determined by comparison with simultaneously run standards of known M_r (Biorad Laboratories, Richmond, CA) that were stained with Coomassie blue.

Animal Experiments

Three 175 g male Sprague-Dawley rats were injected with 15 to 22 mg anti-GEC Ig. Rats were housed in metabolic cages with free access to water at days 0 to 1, 4 to 5, and 11 to 12 for urinary protein determination, which was performed by the sulfosalicyclic acid precipitation method[30] using Lab-Trol (Dade Diagnostics, Aquada, PR) as protein standards. Renal tissue was obtained by wedge biopsy of kidneys under ether anesthesia at day 5 and processed as described below for IF. An additional animal received 11.4 mg anti-GEC IgG intravenously and 5 days later renal tissue was processed for transmission electron microscopy.

Additional studies were performed on rats that received 5.7 mg anti-GEC IgG (1 rat) or 5.0 mg HRP-conjugated anti-GEC IgG (2 rats). Animals were killed at 5 or 3 days after antibody injection, respectively, and the kidneys were processed for immunoelectron microscopy as described below. Rats serving as controls received intravenous injections of 5.7 mg normal rabbit IgG (1 rat) or 5.0 mg HRP-conjugated normal rabbit IgG (1 rat).

Tissue Processing for IF and Electron Microscopy

Direct and indirect IF procedures were performed on tissue snap-frozen in isopentane and cryostat sections were fixed in ether-alcohol and studied as described.[2] Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel Laboratories) was used for direct IF on kidneys of rats that received intravenous injections of anti-GEC and for indirect IF to detect binding of anti-GEC to cryostat sections of normal rat kidney.

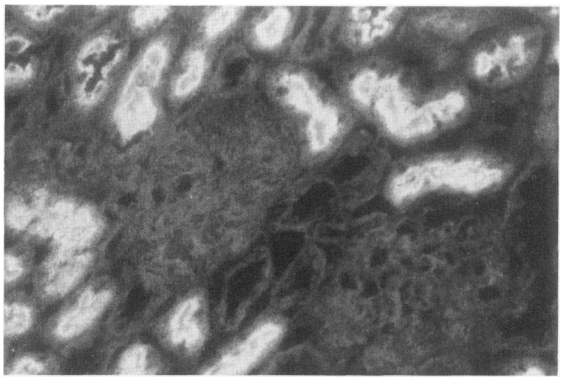

Figure 1. *IF micrograph showing anti-GEC staining of normal rat kidney. Anti-GEC (40 μg/ml) stains BB but glomerular staining is not demonstrable (×400).*

Tissue for routine electron microscopy was fixed by the subcapsular injection of 1.6% paraformaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4.[31] Cubes of cortex were postfixed in 1% buffered osmium tetroxide, dehydrated in graded ethanols followed by propylene oxide, and embedded in epoxy resin. Thick sections were cut and stained with toluidine blue and representative areas thin sectioned, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM 100 CX transmission electron microscope.

Kidney tissue from rats that received intravenous injections of unconjugated anti-GEC IgG or control rabbit IgG were lightly fixed with 1% paraformaldehyde and 0.05% glutaradehyde in 0.1 M phosphate, pH 7.3. Forty-micron-thick frozen sections were obtained on a sliding microtome and these were treated with 0.5% sodium borohydride to neutralize aldehydes introduced by the fixative. Sections were additionally bathed in 1.0 mg/ml bovine serum albumin in PBS and then incubated overnight with HRP-conjugated sheep anti-rabbit IgG, diluted 1:40 in PBS. After labeling with peroxidase conjugates, all sections were washed with PBS, briefly re-fixed in 1.6% paraformaldehyde and 3% glutaraldehyde in sodium cacodylate, pH 7.4.[31] Tissue was then processed for peroxidase histochemistry and electron microscopy as described previously.[32] Kidneys from rats that received intravenous injections of HRP-conjugated anti-GEC IgG or HRP-conjugated normal rabbit IgG were fixed as described above and cubes of tissue were then histochemically processed as before.[32]

Results

Anti-GEC on Normal Kidney

By indirect IF, anti-GEC Ig stained tubular BB at concentrations as low as 40 μg/ml (Figure 1). As is also the case

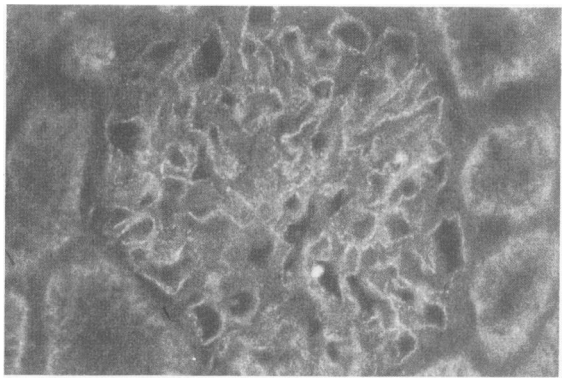

Figure 2. *Glomerulus of a rat 5 days after injection of anti-GEC. Direct IF reveals finely granular capillary wall staining for rabbit IgG (×800).*

with anti-Fx1A, glomerular staining could not be clearly indentified by this technique.

In Vivo *Studies*

Injection of anti-GEC Ig into rats resulted in granular capillary wall deposits of rabbit IgG after 5 days as demonstrated by direct IF (Figure 2). Electron microscopy revealed small electron-dense deposits beneath GEC foot processes and below slit diaphragms (Figure 3a, b). When sections of lightly fixed kidneys from rats that had received rabbit anti-GEC IgG were treated with HRP-conjugated anti-rabbit IgG, discrete deposits of peroxidase reaction product also were found beneath foot processes and slit diaphragms (Figure 4a, b). In addition, coated pits on the soles and sides of foot processes contained reaction product (Figure 4a, b) and some reaction product also was seen intracellularly within GEC (Figure 4b). In contrast, corresponding deposits of HRP reaction product were not present within the subendothelium or mesangial matrix, nor was there any seen intracellularly within endothelial or mesangial cells. In animals that received intravenous injections of direct conjugates of anti-GEC IgG and HRP, reaction product was again found in subepithelial deposits and within what appeared to be lysosomes of GEC (Figure 5a). Peroxidase reaction product was not seen in glomeruli of control rats that received injections of HRP-conjugated normal rabbit IgG (Figure 5b). Significant proteinuria did not develop in animals injected with anti-GEC Ig either in the heterologous or autologous phase (urinary proteins ranged from 2.5 to 12.1 mg/24 hours).

Immunoprecipitation

From ^{125}I-labeled BB, anti-GEC immunoprecipitated a high molecular weight protein (>200 kd), as well as proteins of M_r 120 and 98 kd (Figure 6, lane 3). The high molecular weight protein comigrated with the protein precipitated by rabbit anti-gp330 (lane 1, arrow). Sheep anti-Fx1A also immunoprecipitated these three proteins (lane 2). Protein bands of M_r 43 kd and less were seen in a control immunoprecipitation using normal rabbit Ig (lane 4), and thus probably represent nonspecifically precipitated proteins (possibly including actin, which has an M_r of 43 kd).

As shown in Figure 7, anti-GEC immunoprecipated five proteins of M_r 172, 120, 98, 78, and 42 kd from surface-labeled GEC (lane 2). Anti-Fx1A immunoprecipitated identical proteins as well as proteins of M_r 132, 63, and 45 kd (lane 3). Gp330 was not immunoprecipitated from surface-labeled GEC (lane 4), but was immunoprecipitated

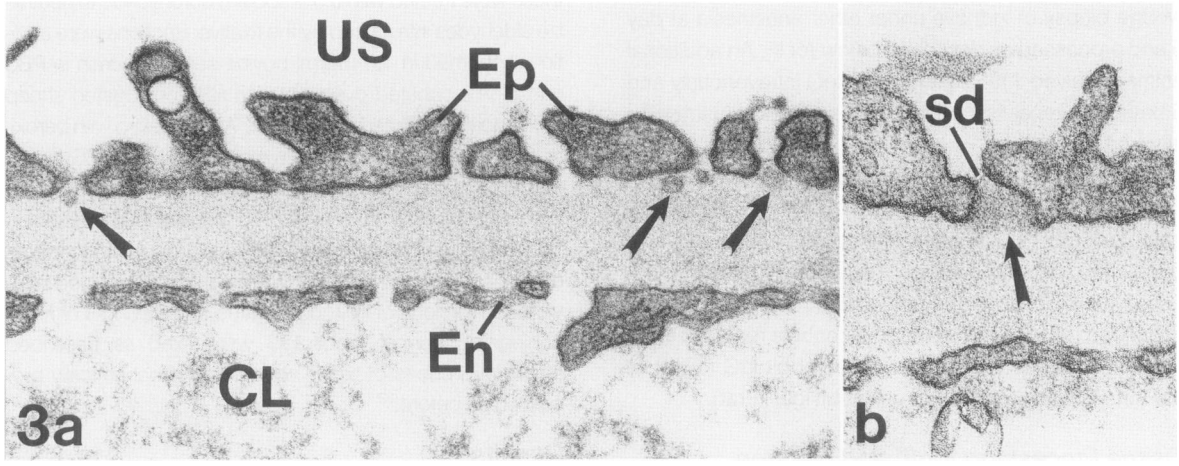

Figure 3. *Standard transmission electron micrographs of glomerular capillary walls from a rat that received anti-GEC IgG 5 days prior to fixation.* **a:** *Several electron-dense deposits can be seen beneath foot processes and slit diphragms in the lamina rara externa of the glomerular basement membrane (arrows). CL, capillary lumen; EN, endothelium; Ep, foot processes of visceral epithelial cells; US, urinary space (×60,000).* **b:** *Higher magnification view of an electron-dense deposit (arrow) in the subepithelium beneath a slit diaphragm (sd) (×78,000).*

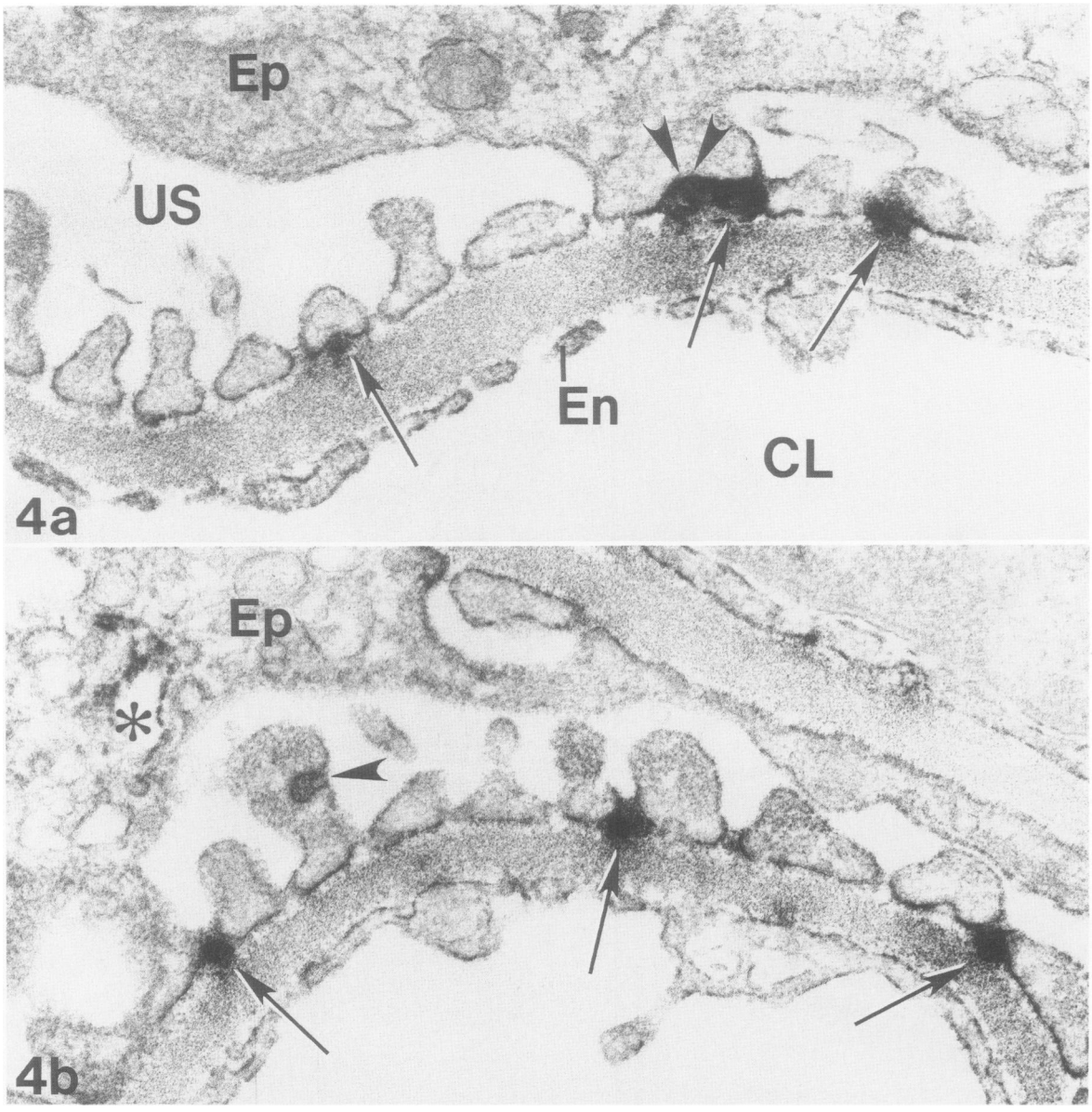

Figure 4. *Electron micrographs of glomerular capillary walls from rats that received rabbit anti-GEC IgG 5 days before fixation. Sections were incubated with HRP-conjugated anti-rabbit IgG.* **a:** *Peroxidase reaction product, which appears black in these micrographs, is present beneath several foot processes (arrows) and also is associated with coated pits (arrowheads). The subendothelium is generally negative. CL, capillary lumen; En, endothelium; Ep, visceral epithelial cell; US, urinary space (×61,000).* **b:** *In addition to reaction product beneath slit diaphragms (arrows), HRP is also present within an endocytic pit on the lateral surface of a foot process (arrowhead) and intracellularly (asterisk) within a visceral epithelial cell (Ep) (×61,000).*

from biosynthetically-labeled GEC with anti-gp330 (Figure 8, lane 5, arrow), anti-Fx1A (lane 3), and anti-GEC (lane 2). Anti-GEC also immunoprecipitated proteins of M_r 120, 108, 78, and 69 kd from biosynthetically-labeled GEC (Figure 8, lane 2).

Discussion

This study shows that antibodies raised against antigens from GEC in culture can produce granular glomerular capillary wall deposits of IgG and subepithelial electron-dense deposits that closely resemble those in PHN, induced by antibodies raised against BB or its purified components. Furthermore, we show that BB and GEC share antigens recognized by anti-GEC and the pathogenic antibodies of PHN, anti-Fx1A, and anti-gp330. This provides additional evidence that the GEC expresses cross-reactive antigens that can serve as the target of *in vivo* antibody binding and subsequent formation of subepithelial immune deposits. Preservation of these antigenic determinants and their continued expression by GEC in long-term cul-

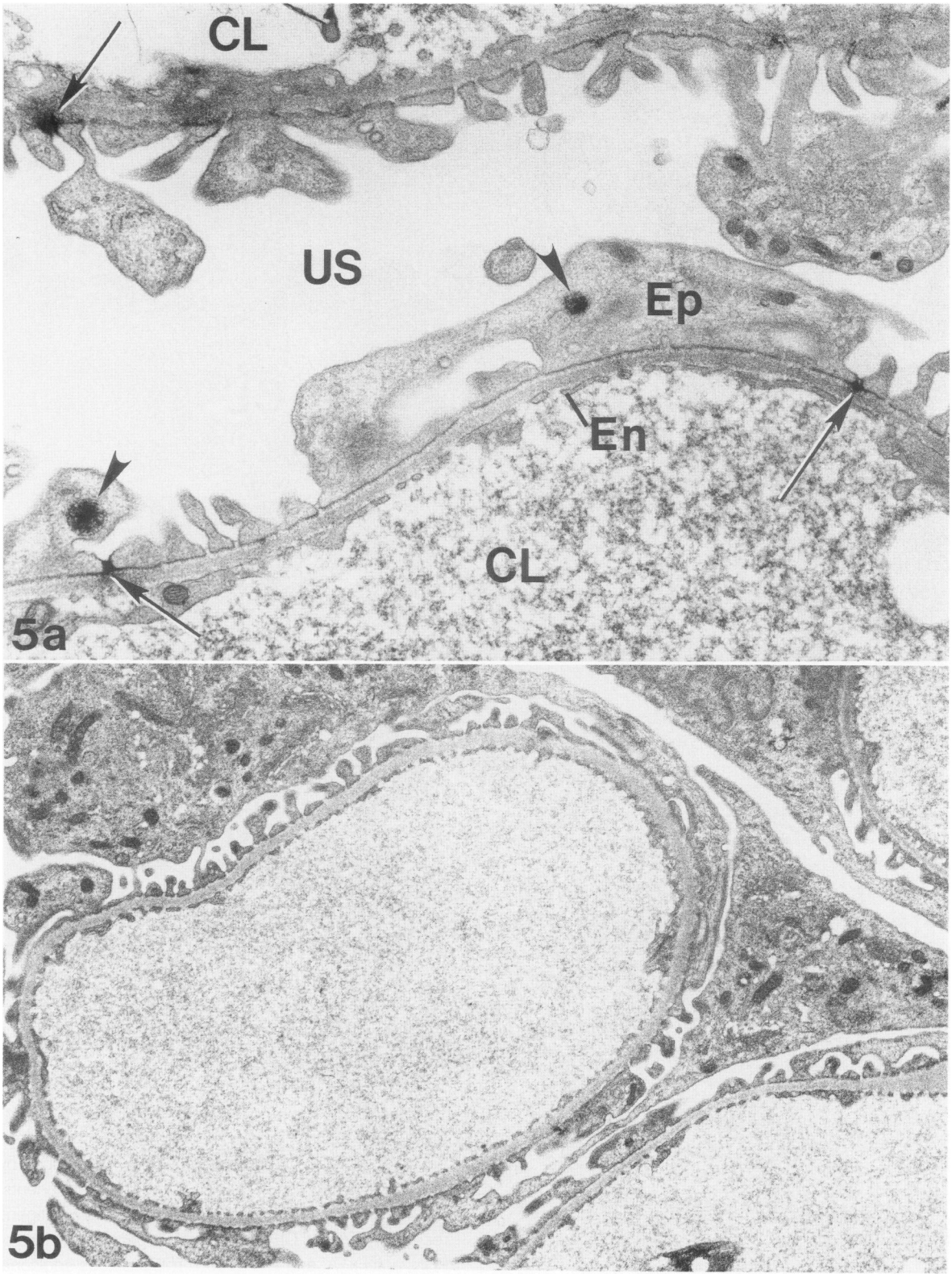

Figure 5. *Immunoelectron micrographs of portions of glomerular capillary loops from rats that received intravenous injections of direct conjugates of HRP and rabbit anti-GEC IgG* (a), *or control HRP-conjugated rabbit IgG* (b), *3 days before fixation.* a: *In rats that received HRP-conjugated anti-GEC IgG, peroxidase reaction product was found in the glomerular basement membrane beneath foot processes and slit diaphragms (arrows), as well as intracellularly within what appeared to be lysosomes (arrowheads) of visceral epithelial cells (Ep). CL, capillary lumen; En, endothelium; US, urinary space (×22,500).* b: *In control rats that received HRP-conjugated normal rabbit IgG, there was no reaction product detected within the glomerular basement membrane or elsewhere within glomeruli (×11,500).*

ture permits the use of these cells for studies of the immunopathogenesis of rat membranous nephropathy.[16-20,23,33]

The findings that antibodies raised against GEC in culture react with BB gp330 and that biosynthetically-labeled GEC produce immunoprecipitable gp330 indicate that gp330 is synthesized by GEC. This is consistent with the immunolocalization of gp330 to biosynthetic organelles in GEC in kidney sections[5] and with *in vivo* studies showing progressive growth of subepithelial immune deposits containing gp330 after anti-gp330 injection, presumably indicating *de novo* synthesis of gp330 by the GEC.[6] The inability to immunoprecipitate gp330 from surface-labeled GEC may reflect absent or low levels of membrane expression of gp330 under basal conditions in culture. Nevertheless, the synthesis of gp330 by GEC in culture may facilitate the characterization of this important molecule.

Although the immunohistochemical features after injection of anti-GEC, including binding to coated pits and subepithelial immune deposit formation, are similar to those seen after injection of anti-gp330,[5,6] the finding of intracellular antibody is unique to this study. This presumably reflects endocytosis of cell surface-bound antibody by the GEC, and may indicate that binding to antigen(s) besides gp330 is required for endocytosis to occur. After endocytosis of antigen and bound antibody, intracellular processing may take place, including dissociation of antigen from antibody, antibody degradation, and exocytosis of antibody and/or antigen (into subepithelial or urinary spaces).[34] Recent preliminary evidence from *in vitro* studies has shown that such pathways may be important in PHN (Quigg RJ, Salant DJ, unpublished observations).[35] Injection of HRP-conjugated anti-laminin into rats also results in some apparent binding on or near the surfaces of GEC, but no endocytosed HRP is found.[32] This suggests that the observed intracellular antibody in our studies is not a universal endocytic response of GEC to subepithelial immune complex formation. Rather, it may represent the effects of one or more antibodies binding to anti-

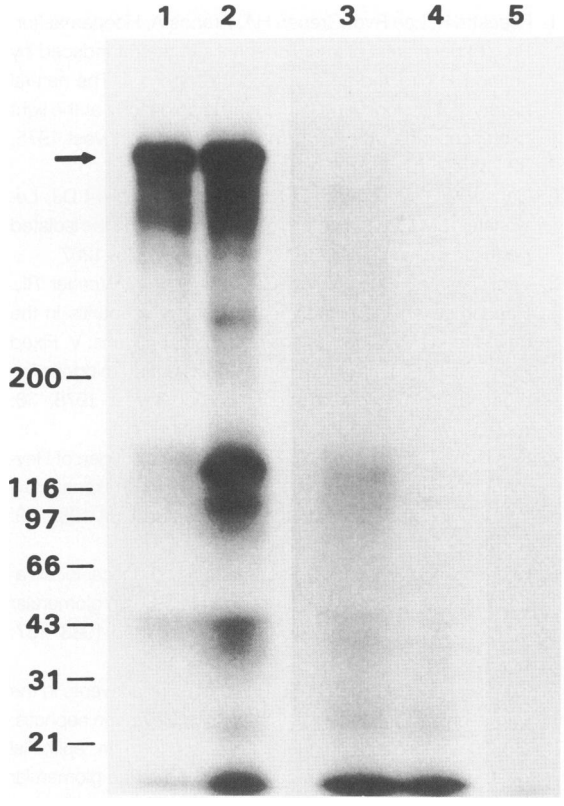

Figure 6. *Autoradiogram of radiolabeled BB proteins after immunoprecipitation with anti-gp330 (lane 1), anti-Fx1A (lane 2), anti-GEC (lane 3), normal rabbit Ig (lane 4), and normal sheep IgG (lane 5), and separation by SDS-PAGE (4 to 12% gradient) under reducing conditions. Anti-gp330 and anti-Fx1A react predominantly with a protein of M_r > 200 kd (arrow). This protein is also recognized by anti-GEC, but to a relatively minor extent. Proteins of M_r 120 and 98 kd are immunoprecipitated by anti-Fx1A and anti-GEC. M_r standards are indicated on the left.*

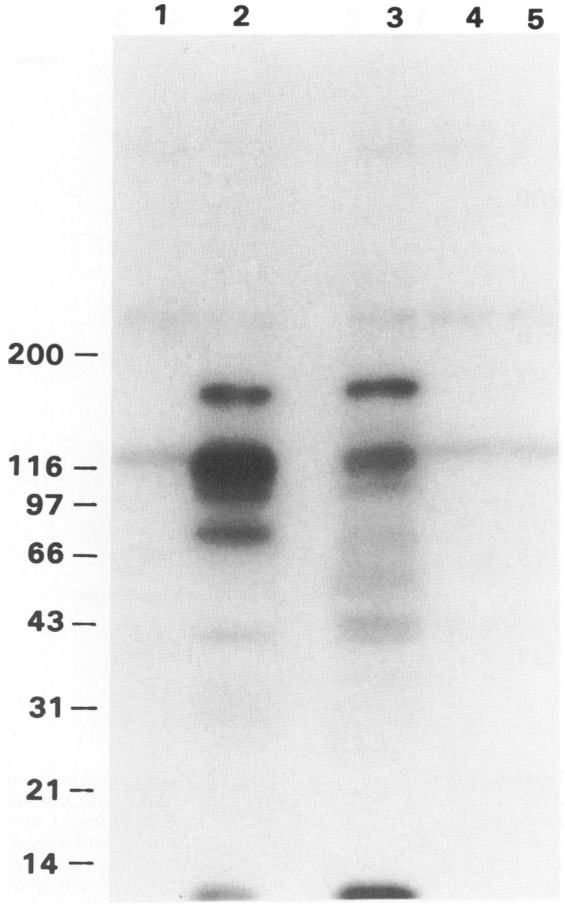

Figure 7. *Autoradiogram of membrane proteins from surface-radiolabeled cultured GEC after immunoprecipitation with normal rabbit Ig (lane 1), anti-GEC (lane 2), anti-Fx1A (lane 3), anti-gp330 (lane 4), and normal sheep IgG (lane 5), and separation by SDS-PAGE (4 to 12% gradient) under reducing conditions. Proteins of M_r 172, 120, 98, 78, and 42 kd are immunoprecipitated by both anti-GEC and anti-Fx1A. There is no specific protein immunoprecipitated by anti-gp330. M_r standards are indicated on the left.*

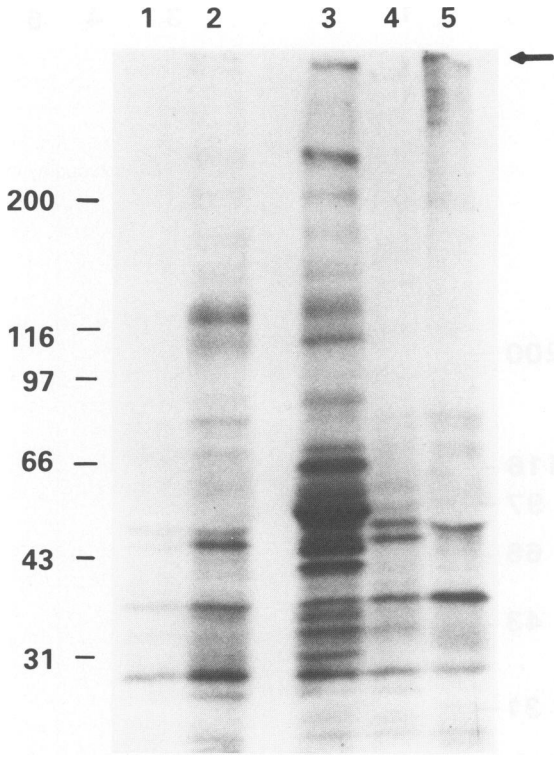

Figure 8. *Autoradiogram of biosynthetically-labeled proteins from cultured GEC after immunoprecipitation with normal rabbit Ig (lane 1), anti-GEC (lane 2), anti-Fx1A (lane 3), normal sheep IgG (lane 4), and anti-gp330 (lane 5), and separation by SDS-PAGE (4 to 12% gradient) under reducing conditions. Anti-gp330 reacts predominantly with a protein of M_r > 200 kd (arrow), that is also identified by anti-Fx1A, and, to a minor extent, by anti-GEC. Proteins of M_r 120, 108, 78, and 69 kd are also immunoprecipitated by anti-GEC. M_r standards are indicated on the left.*

gen(s) that follow an endocytic pathway, perhaps analogous to the internalization of the T3-T cell receptor complex induced on T lymphocytes by monoclonal antibody to T3.[36]

The ability of anti-Fx1A to induce proteinuria is superior to that of anti-gp330, possibly because of interaction of anti-Fx1A with multiple antigens on the GEC.[8] Despite the polyspecific nature of anti-GEC (with antigen specificities similar to anti-Fx1A), injection of anti-GEC into animals did not result in the development of significant proteinuria. This could have been due to the lack of specificity to a "critical" antigen, or, more likely, to the low titer of specific antibody in the anti-GEC preparation (ie, rabbit anti-GEC stained BB at 40 μg/ml whereas sheep anti-Fx1A stained BB at concentrations as low as 10 ng/ml,[2] a 4000-fold difference in titer). Thus, adequate quantities of antibody, necessary to reach a nephritogenic threshold[37] and possibly activate complement,[25] may not have been attained in the *in vivo* studies.

In addition to gp330, anti-GEC indentified proteins of 120 and 98 kd from BB; from GEC similar proteins were immunoprecipitated along with proteins of 172, 78, and 42 kd. The relative importance of these antigens to the development of disease cannot be determined in this study. One potential target antigen expressed *in vivo* by GEC (and also by BB) is dipeptidyl peptidase IV. The M_r of this protein has been reported to be 108[13] and 90 kd.[7] It is possible that the protein of 108 kd immunoprecipitated from biosynthetically-labeled GEC by anti-Fx1A and anti-GEC is dipeptidyl peptidase IV. A 98 kd protein, but not a 108 kd protein, was immunoprecipitated from surface-labeled GEC and BB, potentially indicating that a modification of this molecule exists on the plasma membrane. Clearly there are an increasing number of antigens expressed by GEC being recognized that may be important in the development of PHN or related disorders.[7,8] The use of GEC in culture may allow for further identification and characterization of these proteins.

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