INITIAL EVENTS IN THE FORMATION OF IMMUNE DEPOSITS IN PASSIVE HEYMANN NEPHRITIS

gp330–Anti-gp330 Immune Complexes Form in Epithelial Coated Pits and Rapidly Become Attached to the Glomerular Basement Membrane

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Immunologic mechanisms leading to the deposition of immune complexes in glomeruli are the main causative factors in many renal diseases. Little is known, however, about the molecular mechanisms responsible for the accumulation of immune deposits (IDs)[1] in the glomerulus. To gain insights into this problem we have been studying the formation of IDs in Heymann nephritis (HN), an experimental model of human membranous nephropathy, which is known to be caused by the interaction between an endogenous glomerular antigen and circulating anti–brush border antibodies (1, 2). Previously, we have identified the pathogenic antigen as a large membrane glycoprotein, gp330 (3), and have shown by immunoelectron microscopy that it resides in clathrin-coated pits located both in the proximal tubule brush border and in the glomerular epithelium (4–9). These findings have subsequently been confirmed by others (10–18). Moreover, we presented evidence that when anti-gp330 IgG is injected into normal rats producing so-called passive HN and their kidneys are examined 3 d later, the injected IgG that binds to glomeruli is sometimes found in clathrin-coated pits located at the base of the foot processes of the glomerular epithelium (4–8). Based on these findings we postulated (4–7) that (a) the coated pits on the soles of the foot processes represent the sites where the circulating anti-gp330 IgG and the membrane-associated antigen, gp330, meet; (b) the immune complexes thus formed are subsequently shed into the lamina rara externa of the GBM where they become the nidus of an ID; and (c) the shed immune complexes become stabilized in the lamina rara externa by crosslinking.

We here report results that fully confirm these postulates and allow us to

[1] Abbreviations used in this paper: DAB, diaminobenzidine; DOC, deoxycholate; GBM, glomerular basement membrane; HN, Heymann nephritis; HRP, horseradish peroxidase; ID, immune deposit; PLP, periodate-lysine-paraformaldehyde fixative; TBS, Tris-buffered saline.

reconstruct some of the early events that occur in the formation of subepithelial IDs in passive HN.

Materials and Methods

Animals.   Male Sprague-Dawley rats (200-250 g) were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA, or from the Tierzuchtinstitut der Universitat Wien, Himberg, Austria.

Materials.   Chloramine T, pepstatin A, antipain, leupeptin, benzamidine, DIFP, DNAse (type II), diethylamine, Triton X-100, p-phenylenediamine, diaminobenzidine hydrochloride (DAB, type II), BSA and poly-D,L-lysine hydrobromide were purchased from Sigma Chemical Co., St. Louis, MO; CNBr-activated Sepharose CL-4B and Pharmalytes (pH 3-10) were from Pharmacia Fine Chemicals, Piscataway, NJ; Iodogen was from Pierce Chemical Co., Rockford, IL; sodium-deoxycholate (DOC), Aquacide II, and Azocoll were from Calbiochem-Behring Corp., San Diego, CA; SDS and acrylamide were from Bio-Rad Laboratories, Richmond, CA; ^{125}I was from Amersham Corp., Arlington Heights, IL; CLSPA-collagenase was from Worthington Biochemical Corp. (Freehold, NJ). Heparinase and cationized ferritin, pI 8.6-9.3, were from Miles Laboratories, Inc. (Naperville, IL). F(ab')$_2$ fragments of goat anti-rabbit IgG conjugated to FITC, and FITC-conjugated rabbit anti-goat IgG were obtained from Cooper Biomedical, Inc., Malvern, PA; F(ab) fragments of sheep anti-rabbit and sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP) were from Biosys, Compiègne, France; goat anti-rabbit IgG coupled to 10 nm gold (GAR 10), goat anti-mouse IgG coupled to 5 nm gold (GAM 5), and protein A-gold (5 nm) were purchased from Janssen Life Sciences, Piscataway, NJ. MEM with Eagle's salts was from Gibco Laboratories (Grand Island, NY).

Preparation of Polyclonal and Monoclonal Anti-gp330 IgG.   Polyclonal anti-gp330 IgG was obtained from a rabbit immunized with purified gp330 as described previously (4). This IgG specifically recognizes gp330 and maltase (an antigenically related glycoprotein) by immunoprecipitation (9). Its titer is >2,000 (at 10 mg IgG/ml) by indirect immunofluorescence on cryostat sections of normal rat kidney. Mouse monoclonal anti-gp330 IgG was obtained from clone D$_1$55F2. It recognizes only gp330 by immunoprecipitation (9). Normal rabbit IgG was purified from sera of nonimmunized rabbits.

Iodination of Rabbit IgG.   IgG was labeled with ^{125}I by the iodogen procedure (19) (sp act, 250 × 10^6 cpm/mg). Nonimmune rabbit IgG was similarly labeled (sp act, 120 × 10^6 cpm/mg) as a control. The nonimmune IgG was depleted of anti-gp330 crossreactivity by circulating it for 12 h over a column containing purified gp330 immobilized to Sepharose 4B by CNBr.

Induction of Passive HN.   20 rats were infused for 1 min intravenously via the jugular or saphenous vein with 3-5 mg rabbit anti-gp330 IgG, and their kidneys were collected as described below after 15 (4 rats) or 30 min (2), 1 (4) or 2 h (2), or 1 (2), 3 (4), or 8 d (2), and they were processed for immunofluorescence and immunoelectron microscopy. 12 rats were similarly injected with a mixture of 2 mg of ^{125}I-labeled rabbit anti-gp330 IgG and 3 mg nonradiolabeled anti-gp330 IgG suspended in 0.5-0.7 ml PBS. The animals were killed after 15 min (4 rats), 1 h (4), or 3 d (4), and their kidneys were collected and used for isolation of glomeruli and GBMs. Seven additional rats were injected with 5 mg ^{125}I-labeled nonimmune rabbit IgG, and their kidneys were collected after 15 min (2 rats), 1 h (2), and 3 d (3).

Preparation and Processing of Tissue for Immunocytochemistry.   Kidneys of rats injected with anti-gp330 IgG and controls were perfused via the abdominal aorta with 25-30 ml of MEM for 5 min at 20°C. The left renal artery was ligated, the left kidney was removed, and tissue samples were frozen and stored in liquid N$_2$ until used for immunofluorescence. The right kidney was perfused with 15-20 ml of periodate-lysine-paraformaldehyde fixative (PLP) (20) for 3 min. Small blocks were prepared from the cortex, fixed further by immersion in PLP for 4-6 h at 20°C, after which they were cryoprotected in 10% DMSO, and frozen in liquid N$_2$-cooled isopentane until processed for immunoperoxidase (4, 8, 9).

Immunofluorescence. Cryostat sections (4 μm) were incubated with FITC-goat anti–rabbit F(ab')₂ (to detect rabbit IgG) for 20 min, washed in PBS (3 × 15 min), and mounted in glycerol. In the case of the 15-min group (Fig. 1*A*), sections were also incubated with FITC-rabbit anti–goat IgG (20 min) to enhance the fluorescent signal. Sections were examined in a Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY), equipped with epifluorescence illumination.

Immunoperoxidase. Cryostat sections (20 μm) prepared from PLP-fixed kidney samples were incubated either in (*a*) sheep anti–rabbit Fab-HRP (diluted 1:50) for detection of bound rabbit IgG, or (*b*) in monoclonal anti–gp330 IgG (10 μg/ml) followed by sheep anti–mouse Fab-HRP for detection of endogenous gp330. The sections were then fixed in 1.5% glutaraldehyde in 100 mM cacodylate buffer, incubated in DAB-medium (0.1% DAB, 0.01% H₂O₂) for 1–3 min, postfixed in ferrocyanide-reduced OsO₄, and embedded in Epox as described (4, 9). Ultrathin sections were stained with alkaline lead citrate and examined in a Philips 301 or a Zeiss 9S electron microscope.

Isolation of Glomeruli and GBMs. Kidneys of rats injected with ^{125}I-labeled IgG were perfused with MEM for 2–3 min at 20°C and then removed and frozen in liquid N₂. Those obtained from all rats of a given time point were pooled, and the glomeruli were isolated by sieving, yielding a fraction of >80% purity. A small aliquot was processed for morphological studies, and another was saved and counted in a Biogamma Counter (Beckman Instruments, Inc., Fullerton, CA). The remaining glomeruli of each experimental group were mixed with those prepared from 8–10 normal (noninjected) rat kidneys as a carrier.

GBMs were purified (21) by extracting isolated glomeruli with distilled water (1 h at 20°C), followed by digestion with 1 U/ml DNAse in unbuffered 1 mM CaCl₂ (30 min). The suspension was then centrifuged (1 min at 10,000 *g* in a Microfuge), and the pellet was resuspended (2 h) in 1% DOC in Tris-buffered saline (TBS), pH 7.2, with 0.1% BSA, containing protease inhibitors (1 mM DIFP, 5 mM pepstatin A, 5 mM antipain, 5 mM leupeptin, 10 mM benzamidine, 1 mM iodoacetamide, and 5 mM EDTA). The supernatants obtained were saved and counted to determine the cpm released at each step in the GBM purification procedure. The resultant pellet containing the GBMs was subsequently washed (5×) in TBS with 0.1% BSA by repeated pelleting (30 s in a Microfuge) and resuspension. The isolated GBMs were then either counted for bound radioactivity or processed for immunolabeling.

Quantitation of the Effects of Various Treatments on Release of Rabbit IgG Bound to the GBM. Aliquots of unfixed GBMs isolated from the 3 d group were incubated in suspension in Eppendorf vials in: (*a*) 1 or 2 M NaCl for 2 h at 20°C; (*b*) 0.1 U heparinase in 0.1 M acetate buffer, pH 6.0, containing protease inhibitors (1 mM pepstatin, 1 mM antipain, and 10 mM benzamidine) for 12 h at 37°C; (*c*) 0.01 mg collagenase in 200 mM NaCl, 5 mM CaCl₂, 100 mM Tris-HCl, pH 7.5, containing the same protease inhibitors as in *a* for 6 and 12 h at 37°C; (*d*) 100 mM diethylamine in distilled water (pH >11); or (*e*) 1 M acetic acid (pH <3.0) for 30 min at 20°C. The GBMs were pelleted by centrifugation (5 min in a Microfuge) and the radioactivity of the supernatant and pellet was determined.

Double Immunolabeling of Isolated GBMs. Isolated GBMs were prepared for immuno-electron microscopy from rats given ^{125}I-labeled anti-gp330 or nonimmune IgG by two methods: Freshly prepared, unfixed GBMs were suspended in PBS mixed 1:1 with 3% agarose in PBS, and cast into a thin film (22). Small strips (2 × 3 mm) were cut from the agarose films and incubated 2 h at 20°C with 5 nm gold–protein A conjugate diluted 1:5 in PBS containing 1% OVA to localize rabbit IgG. After washing (3 × 2 h) in PBS, they were then processed by the immunoperoxidase procedure for localization of endogenous gp330 as described above.

Alternatively, GBMs were suspended in PBS and 50-μl aliquots were dropped onto glass slides precoated with poly-L-lysine. After incubation for 2 h at 4°C, the unbound material was removed by rinsing with PBS, and the slides were immersed in PLP-fixative for 30 min. After washing (3 × 20 min) in PBS with 1% OVA, the immobilized GBMs were incubated sequentially in (*a*) 25 mM glycine in PBS for 15 min (to quench free

aldehyde groups; (b) 10 nm gold-goat anti–rabbit IgG in PBS containing 1% OVA for 6 h at 4°C (to detect the injected rabbit IgG); (c) monoclonal anti-gp330 IgG (10–15 μg/ml) in PBS for 6 h at 4°C; and (d) 5 nm gold–goat anti–mouse IgG for 6 h (to detect endogenous gp330). After washing (4 h) in PBS, the samples were fixed in 2.5% glutaraldehyde (1 h), 100 mM cacodylate buffer, pH 7.2, postfixed in 1% OsO₄ (1 h) in the same buffer, and embedded in Epox.

Controls consisted of incubations in which the monoclonal anti-gp330 IgG was omitted, or the specimens were preincubated in goat anti–rabbit IgG (2 mg/ml).

Quantitation of IDs in Tissue Sections and in Isolated GBMs.   Electron micrographs were taken (×25,000) of two to four glomeruli from each kidney in specimens reacted for rabbit IgG by immunoperoxidase. All the capillary loops of a given glomerulus were photographed, and the following parameters were determined for each experimental group: (a) the number of IDs/100 μm of GBM, (b) the number of IDs located under the foot processes vs. those located beneath the slit diaphragms, and (c) the number of foot processes per micrometer GBM.

The number of IDs/100 μm GBM was also determined in GBMs isolated from each experimental group and processed for immunogold labeling. In addition, the number of 10-nm (indicating rabbit IgG) and 5-nm (indicating gp330) gold particles present in the IDs were counted and their ratio was calculated.

Reconstruction of IDs from Serial Sections—Ribbons of 60–80-nm sections prepared from specimens reacted by immunoperoxidase for rabbit IgG were collected on carbon-formvar–coated grids, and identical areas of serial section were photographed (×25,000). The micrographs were then overlayed with transparent paper, and the contours of individual IDs were traced. The three-dimensional shape of 45 IDs was reconstructed by superimposing the tracings of each ID, and the relationship of individual IDs to coated pits and slit diaphragms was determined.

Immunoprecipitation with IgG Eluted from IDs.   Glomeruli isolated from rats injected with 10 mg unlabeled rabbit-anti gp330 IgG 3 d earlier were extracted with 20 mM citrate buffer, pH 3.2, at 37° for 30 min, followed by extraction with the same buffer, pH 2.8, as described previously (3, 23). The eluted IgGs were used for immunoprecipitations from ¹²⁵I-labeled microvillar fractions solubilized in RIPA-buffer (3).

Isoelectric Focusing of IgG.   GBMs were isolated from a control rat and from two rats 1 h and 3 d after injection with ¹²⁵I-labeled anti-gp330 IgG; they were then extracted with 100 mM diethylamine (pH >11) for 15 min at 20°C to release the bound IgG (24). The supernatants containing the extracted IgG were neutralized with 1 M HCl, dialyzed against TBS, and incubated with 200 μl protein A–Sepharose 4B (1 h at 4°C). After washing (10 ml TBS), the IgG bound to the protein A beads was released with 1 M acetic acid, containing 1 mg/ml of nonradioactive rabbit IgG as a carrier. The fractions were neutralized with 1 M NaOH, lyophilized, and redissolved in 20 μl distilled water. The isoelectric points of the eluted anti-gp330 IgG and of the radioiodinated and nonradioiodinated anti-gp330 IgG used for injection were determined by isoelectric focussing in a Bio-Rad Laboratories flatbed electrophoresis apparatus with Pharmalytes establishing a pH gradient of 2.9–9.5. Cationized ferritin (pI 8.6–9.5) and BSA (pI 4.5) were used as standards. One lane of the agarose gel was cut into 0.5-cm strips, which were incubated in 1 ml distilled water (1 h) to extract the ampholytes, and the pH of the extracts was measured. The agarose gels were fixed and stained in 25% methanol–7% acetic acid with 0.1% Coomassie blue, dried, and exposed for autoradiography.

Results

IDs Originate in Coated Pits and Become Associated with Slit Diaphragms.   The formation of glomerular IDs was studied by immunofluorescence and by immunoelectron microscopy in rats killed at 15 min to 8 d after the injection of rabbit anti-gp330 IgG. As early as 15 min after the injection, a very fine granular pattern of staining for rabbit IgG was detected by immunofluorescence along the GBM (Fig. 1A). At 1 h and 3 d the size of the IDs and their staining intensity
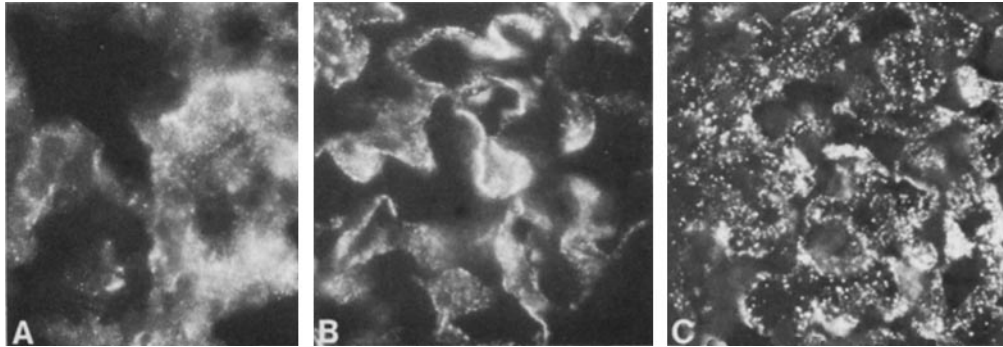
FIGURE 1.   Detection of rabbit IgG in rat glomeruli by direct immunofluorescence at 15 min (A), 1 h (B), and 3 d (C) after injection of 5 mg ^{125}I-labeled, rabbit anti-gp330 IgG. At 15 min, rabbit IgG is distributed in a faint granular pattern along the GBM (A). The sizes of the granular deposits become larger with time (B and C). × 650.

were increased (Fig. 1, B and C). There was no staining of any structure other than the GBM. These findings are in agreement with previous studies (1, 2), in which granular deposits of anti-Fx1A-IgG were observed as early as 5–10 min after injection.

When rabbit IgG was localized by immunoperoxidase at the electron microscope level, at 15 min (Figs. 2A and 3, A–C) or 1 h (Fig. 3, D and E) after antibody injection the bound IgG was found largely or exclusively in coated pits at the base of the epithelial foot processes. It is precisely in this location that the antigen, gp330, is known to reside (4–10, 12, 15). Anti-gp330 IgG was not detected along the remaining (noncoated) areas of the epithelial plasmalemma at the base of the foot processes or in any other location.

After 1 d, some of the IgG was still seen in basally located coated pits which were enlarged and were filled with reaction product (Fig. 3, F and G), but as the IDs enlarged in size they were found with increasing frequency under the slit diaphragms. As before, only some of the coated pits contained IDs (Fig. 3F). At 3 d the IDs were larger and they were found more often under the slit diaphragms (Fig. 4). At 8 d the relationship of IDs to coated pits was seldom evident in individual sections, and most of the large IDs were found under the slit diaphragms or beneath noncoated regions of the epithelial cell membrane at the base of the foot processes (Figs. 2B and 3H).

The number of foot processes per 100 μm GBM remained fairly constant up to 3 d (280 at 15 min; 230 at 3 d), but was slightly decreased at 8 d (190/μm GBM). The average size of the foot processes thus increased from 0.36 μm (15 min) to 0.53 μm (8 d).

No DAB-reaction product was seen in the kidneys of rats injected with normal rabbit IgG.

Serial Sectioning Reveals that IDs Remain Associated with Coated Pits.   The three-dimensional shapes and relationships of 45 different IDs were reconstructed from serial sections such as those shown in Fig. 4 prepared from immunoperoxidase preparations of rats given rabbit anti-gp330 IgG 3 d before killing. The results demonstrated that the size and shape of the IDs varied considerably; they ranged from small globules to large complicated structures with openings
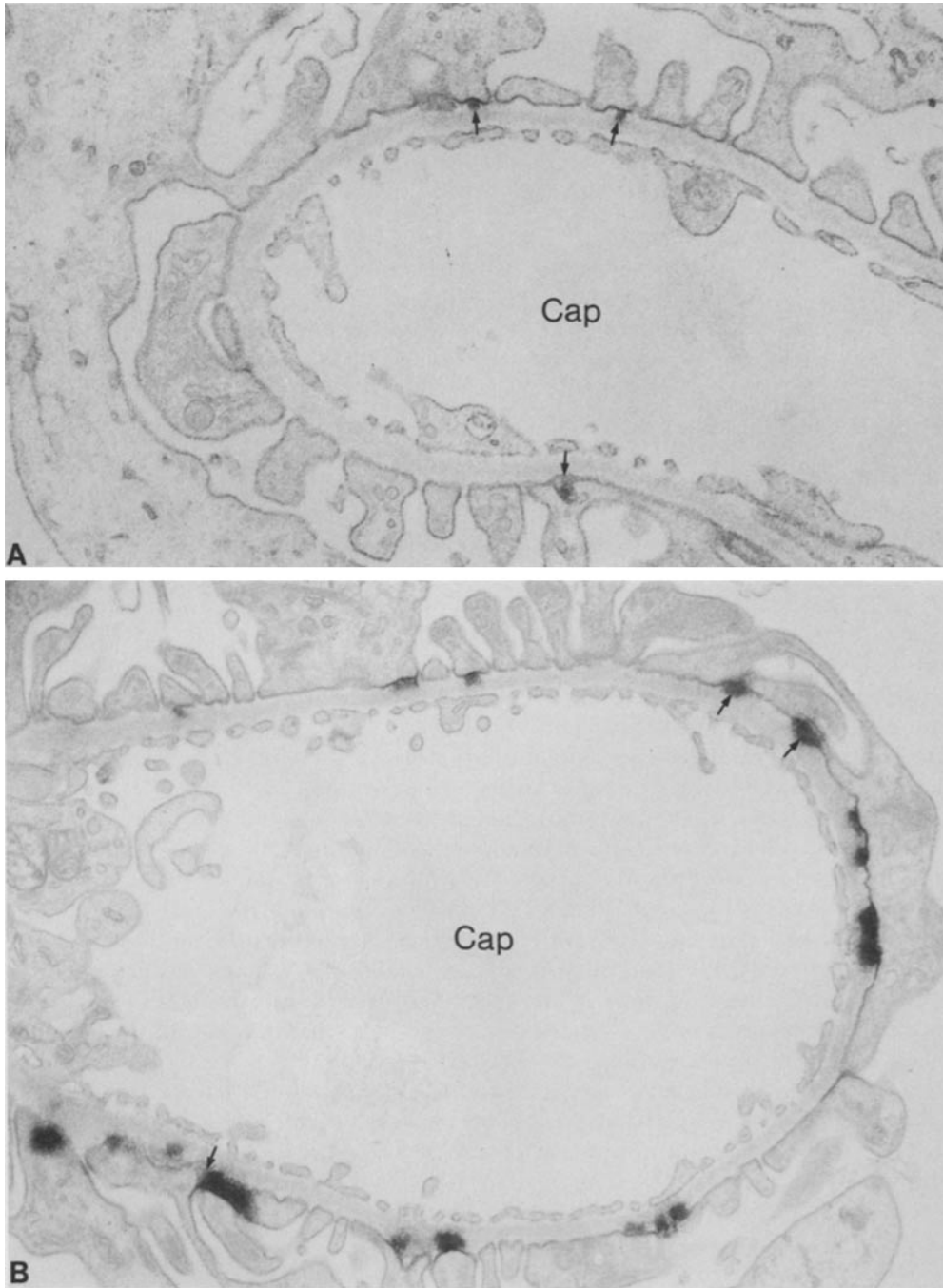
FIGURE 2.   Direct immunoperoxidase localization of rabbit IgG in glomeruli of rats injected with 5 mg of rabbit anti-gp330 IgG and fixed at 15 min (A) or 8 d (B) after injection. At 15 min, three small IDs are seen in the lamina rara externa (arrows) at the base of the epithelial foot processes of this capillary (cap). At 8 d the deposits are larger, more numerous, and are often found in association with slit diaphragms (arrows). Note the absence of reaction product on the endothelial cell surfaces and in the lamina rara interna. Cryostat sections were incubated in sheep anti–rabbit Fab-HRP conjugate followed by the DAB reaction. × 18,000.
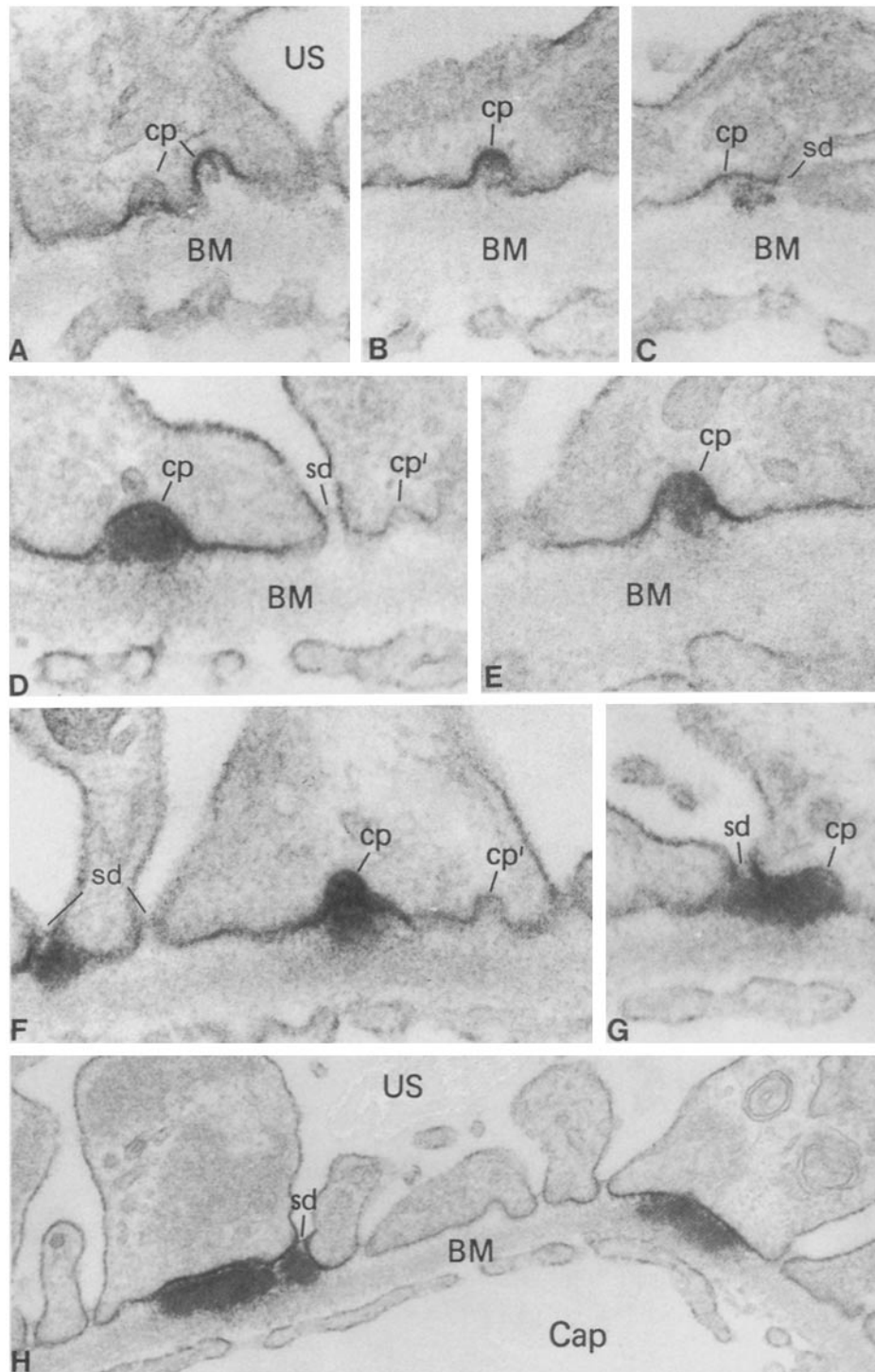
FIGURE 3. Gallery of IDs located along the cell membrane at the base of the epithelial foot processes in tissues fixed at various intervals (15 min to 8 d) after injection of anti-gp330 IgG as in Fig. 2. At 15 min (A–C), rabbit IgG is detected exclusively in coated pits (cp). Occasionally, such coated pits are located near a slit diaphragm (sd in C). After 1 h (D and E), the deposits are larger and still typically associated with coated pits. Some coated pits are devoid of rabbit IgG (cp' in D). After 1 d (F and G), the IDs are increased in size, and some are found under the slit diaphragms (sd), as well as in coated pits. As at 1 h, some coated pits do not contain rabbit IgG (cp' in F). At 8 d (H), the IDs are still larger and are found in the lamina rara externa under both the slit diaphragms (sd) and the noncoated regions of the foot processes. A–G, × 45,000; H, × 30,000.
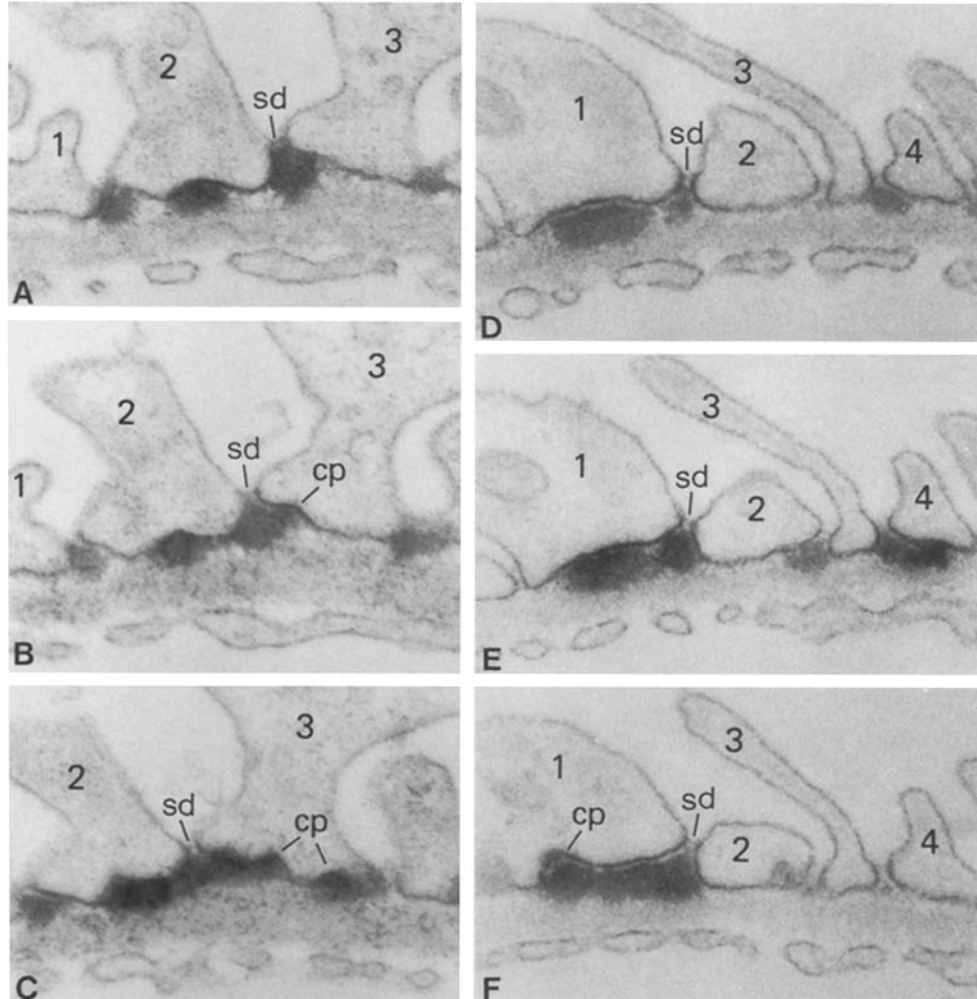
115

FIGURE 4.   Serial (not consecutive) sections through two IDs (*A–C* and *D–F*) in glomeruli of rats given rabbit anti-gp330 IgG 3 d before killing and processed as in Figs. 2 and 3. Individual foot processes are numbered to facilitate their identification. In both series, IDs are present that seem to be located under the slit diaphragms (*sd*) in a given section. However, when followed in serial sections, it is apparent that these IDs are actually extensions of larger deposits that at some level are in contact with a basally located coated pit (*cp*). × 30,000.

through which the foot processes still contacted the GBM (Fig. 5). Although the majority of IDs appeared to be located under the slit diaphragms in individual sections at this time point, when followed in serial sections (Fig. 4) it was obvious that all of the 45 IDs analyzed made contact with a coated pit or a larger clathrin-coated area of the basal epithelial plasma membrane at some level.

The Number of IDs Increases with Time.   Counts of IDs in kidney sections (Table I) indicated there are ~17/100 μm GBM at 15 min after injection, their number remains fairly constant up to 1–2 h, and increases to ~90/100 μm GBM at 3 d.

Immune Deposits Remain Bound to Isolated GBM.   When GBMs isolated from
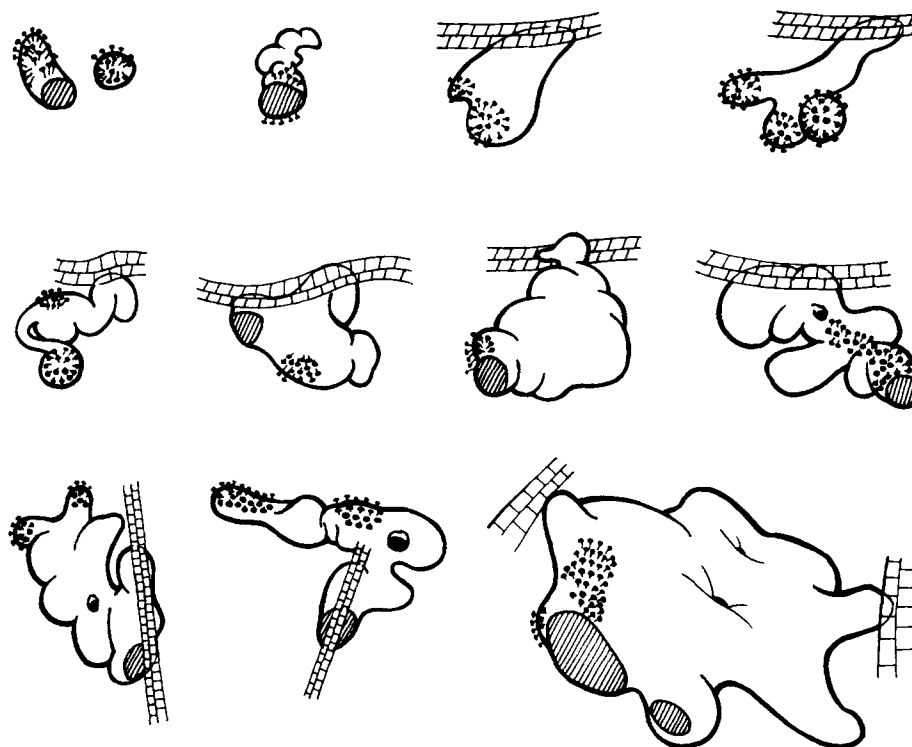
FIGURE 5. Three-dimensional shapes of a number of IDs, as reconstructed from serial sections. The ladder-like structures indicate the position of the slit diaphragms, and the pins indicate the location of coated regions of the epithelial cell membrane. Each ID, regardless of its size, was found to contact a region of coated membrane surface at some level. × 45,000.

TABLE I

Number of Immune Deposits in Intact Kidney Tissue and in Isolated GBMs at Various Time Points after Injection of Anti-gp330 IgG

Time after injection of anti-gp330 IgG	n*	Number of immune deposits per 100 μm GBM[‡]	
		Intact tissue	Isolated GBMs
15 min	4	16.7	15.2
30 min	2	21.3	18.6
1 h	4	12.6	14.4
2 h	2	17.7	ND
1 d	2	30.7	ND
3 d	4	90.8	84.7
8 d	2	66.5	ND

* n, Number of animals per group.
[‡] For each group IDs in 600–1000 μm of GBM were counted.

rats injected with anti-gp330 IgG were examined by electron microscopy, IDs were seen to remain attached and coisolated with them at every time point.

When the number of IDs per 100 μm GBM found in isolated GBMs was compared with the number found *in situ*, there was no significant difference at
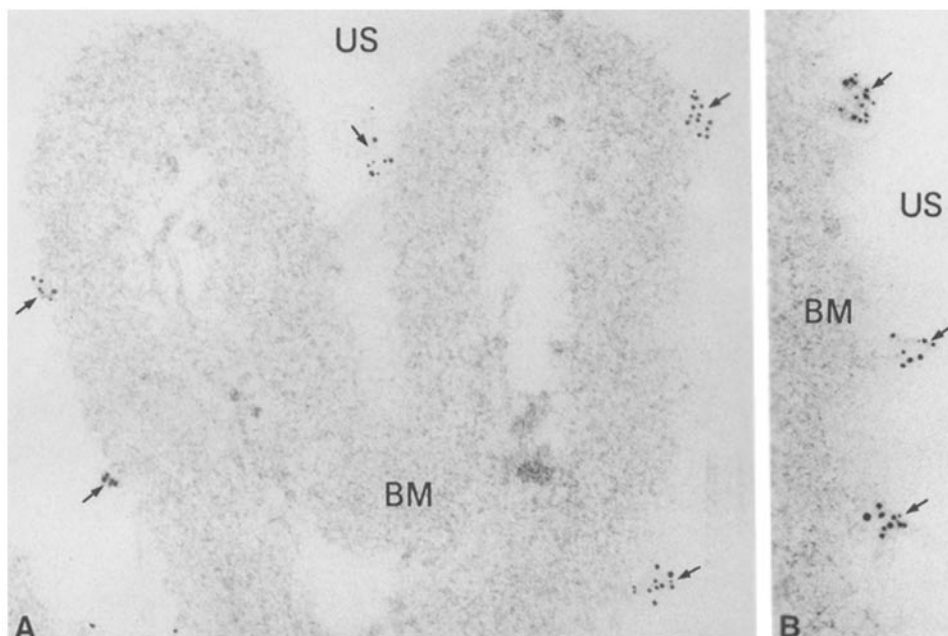
FIGURE 6. GBMs isolated from the kidneys of rats given rabbit anti-gp330 IgG 15 min before killing, demonstrating that IDs remain attached and coisolate with them. The GBMs were fixed, immobilized on poly-lysine-coated glass slides and incubated with goat anti–rabbit IgG coupled to 10 nm gold (to localize the bound rabbit IgG), followed by monoclonal anti-gp330 IgG and goat anti–mouse IgG coupled to 5 nm gold (to localize gp330). Five IDs (arrows) are seen attached to the isolated GBM on the left, and three are seen on the right. Note that most (~90%) of the gold particles are 5 nm in size, and relatively few (lowest arrow in B) are 10 nm, indicating that the antigen gp330 predominates in these early IDs. (US) urinary space, (BM) glomerular basement membrane. × 85,000.

any time point (Table I). This indicates that (a) little or no loss of morphologically recognizable IDs is brought about by the treatments to which GBMs are exposed during their isolation (hypotonic lysis, DNAse digestion and extraction in non-ionic detergent [DOC]), and (b) the IDs are firmly bound to the GBM as early as 15 min after antibody injection.

Both gp330 and Anti-gp330 IgG Can Be Detected in IDs by Immunoelectron Microscopy. Gp330 could be detected in IDs associated with isolated GBMs by the immunogold procedure at all time points (Figs. 6 and 7) and by immunoperoxidase at later time points (Fig. 8). Moreover, after double-immunolabeling for gp330 (5 nm gold) and rabbit IgG (10 nm gold), both the antigen and the antibody could be simultaneously demonstrated in the IDs attached to the isolated GBM at all time points (Figs. 6 and 7). The gold particles did not penetrate the entire depth of most IDs when immunolabeling was done on aldehyde-fixed GBMs. Counts of the two sizes of gold particles indicated that 5-nm gold predominated at 15 min after injection (Fig. 6), whereas equal numbers of 5-nm and -10 nm gold particles were observed after 3 d (Fig. 7).

Gold particles were absent from the GBMs isolated from controls given nonimmune IgG.

These findings indicate that (a) both the antigen and the antibody remain with
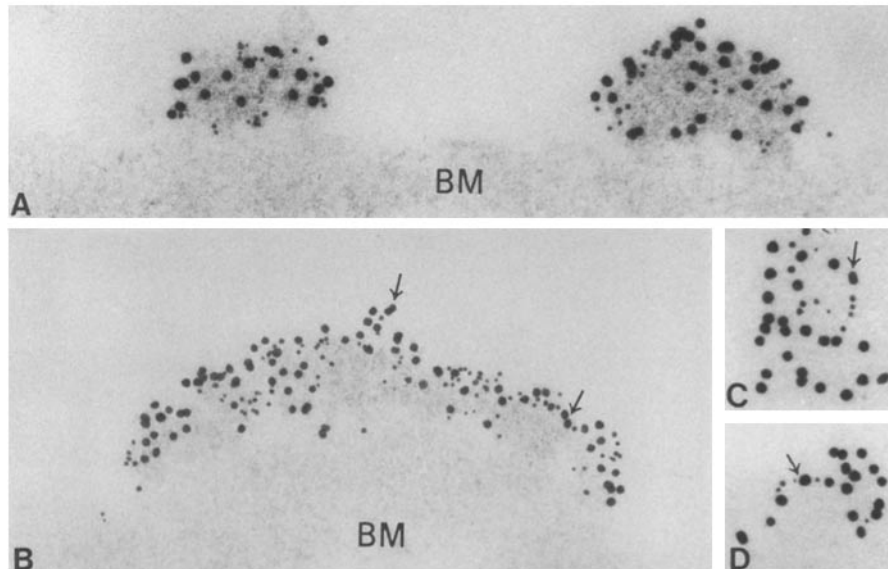
FIGURE 7. Immunogold labeling of GBMs isolated 3 d after anti-gp330 IgG injection and processed as in Fig. 6. At this time IDs are attached to the GBM via a broad base, and the antigen/antibody ratio is reduced: >50% of the particles present are 10 nm in size (indicating rabbit IgG), and the remainder are 5 nm (indicating gp330 antigen). Occasionally, the two sizes of gold particles are found lined up in an alternating pattern (arrows). A, × 110,000; B, × 95,000; C and D, × 120,000.

the IDs during the GBM isolation procedure and (b) initially the antigen/antibody ratio is higher than at later time points.

Quantitation of Rabbit IgG Bound to Isolated GBMs. At 15 min and 1 h after injection of the ^{125}I-labeled IgG, 75 and 60%, respectively, of the counts were lost from glomeruli during the GBM isolation procedure (Table II). By contrast, after 3 d only ~10% was lost, and >90% of the IgG remained bound to the GBMs. In controls given gp330-depleted IgG, much less IgG remained associated with the isolated glomeruli, and >80% of it was released during the GBM preparation procedure at all time points.

When GBMs isolated from the 3-d experimental group were treated with 1–2 M NaCl or were digested with heparinase or collagenase, very little radiolabeled IgG was released (Table III). However, when the isolated GBMs were incubated in 100 mM diethylamine (pH >11) or 1 M acetic acid (pH <3.0), conditions that are known to dissociate immune complexes (23–24), >65% of the radiolabeled IgG was released.

Collectively, these findings together with the immunogold localization studies suggest that at 15 min to 1 h after anti-gp330 IgG injection, much of the IgG (60–75%) is loosely bound and is lost during GBM isolation, whereas after 3 d virtually all of it is firmly bound to the IDs. Some of the firmly bound IgG (65%) can be eluted by dissociating the antigen-antibody complexes, but a significant amount (35%) cannot be eluted by any of the procedures tried.

Immune Deposits Contain Anti-gp330 IgGs with Acidic Isoelectric Points. When
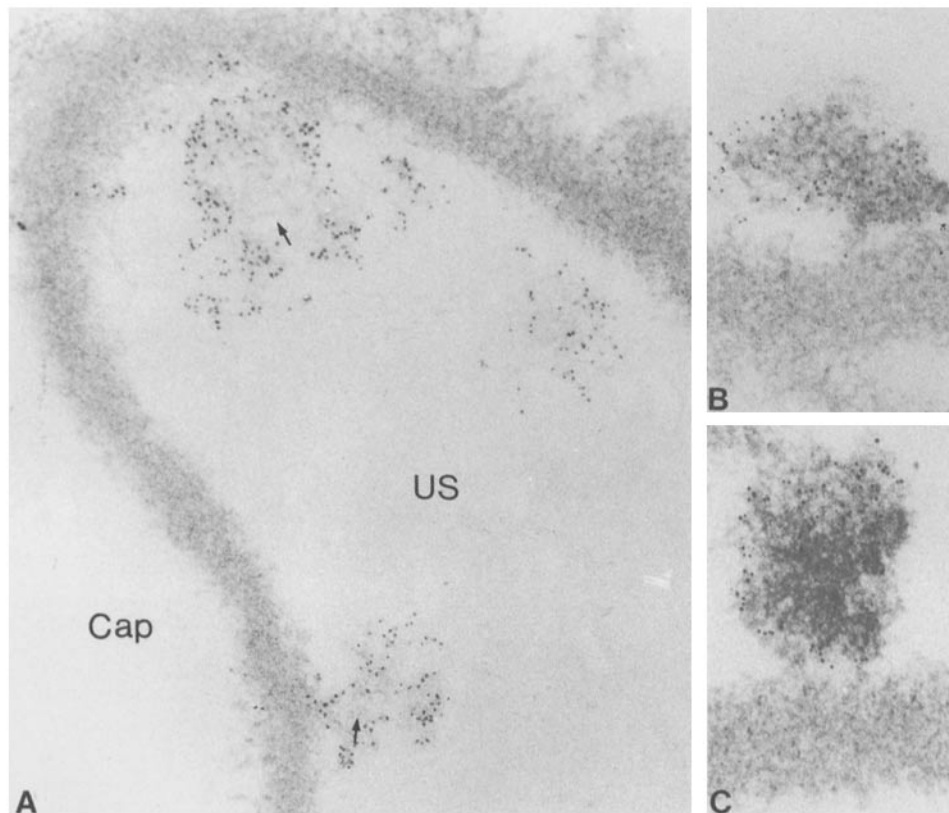
FIGURE 8.   Double-immunolabeling for gp330 and rabbit IgG of IDs attached to isolated
GBMs at 1 h (A), 3 d (B) or 8 d (C). Unfixed, GBMs were embedded in agarose and doubly
incubated in monoclonal anti-gp330 detected by indirect immunoperoxidase (to label gp330)
and in 5 nm gold–protein A conjugate (to label bound anti-gp330). The presence of bound
IgG is demonstrated by the presence of gold particles in all IDs. gp330 is not detected with
the peroxidase technique at 1 h (probably because the DAB reaction product is lost by
diffusion), but it is readily detected in the more compact IDs seen at 3–8 d. A fibrillar
substructure is visible in the less compact IDs seen at 1 h (arrows). A, × 32,000; B and C,
× 85,000.

the IgGs that bound to glomeruli from rats given rabbit anti-gp330 3 d earlier
were eluted and used for immunoprecipitation, only gp330 was precipitated
(data not shown). Similar results were obtained previously (3) with IgGs eluted
from glomeruli of rats with active HN induced by immunization with gp330. To
find out whether the bound anti-gp330 IgGs belong to anionic or cationic
subclasses, we compared the isoelectric points of the eluted IgGs with those of
the injected native (nonradiolabeled) and radiolabeled anti-gp330 IgG. The
latter showed a broad range of isoelectric points from pI 5.0 to 8.5 (Fig. 9, A
and B). The pattern was identical with both native IgG (A) and iodinated (B)
anti-gp330, indicating that the iodination of anti-gp330 did not substantially
influence its isoelectric points. When the isoelectric points of the IgGs eluted
from isolated GBMs of either 1-h and 3-d injected animals were determined, two
major subspecies were observed with acidic isoelectric points of ~5.0 and 6.1
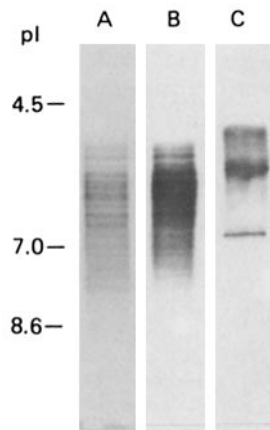
pl

A   B   C

4.5—

7.0—

8.6—

FIGURE 9. Isoelectric focusing of rabbit anti-gp330 IgG. (A) Coomassie blue–stained, protein A–purified, rabbit anti-gp330 IgG. (B) Autoradiogram of the same fraction as in A after labeling with ^{125}I. (C) Autoradiogram of IgG eluted from isolated glomeruli of rats killed 3 d after injection of the fraction shown in B and purified on protein A–Sepharose. The range of isoelectric points of native (A) and radiolabeled (B) IgG are very similar, whereas the rabbit IgG eluted from glomeruli of injected rats (C) is composed mainly of IgGs with acidic pIs (5.0–6.1). The line in the region of pI 7 in C is where the sample was loaded onto the gel.

A   B   C   D   ＼—ve

T Clathrin coat
♀ gp 330
Y anti gp 330 IgG
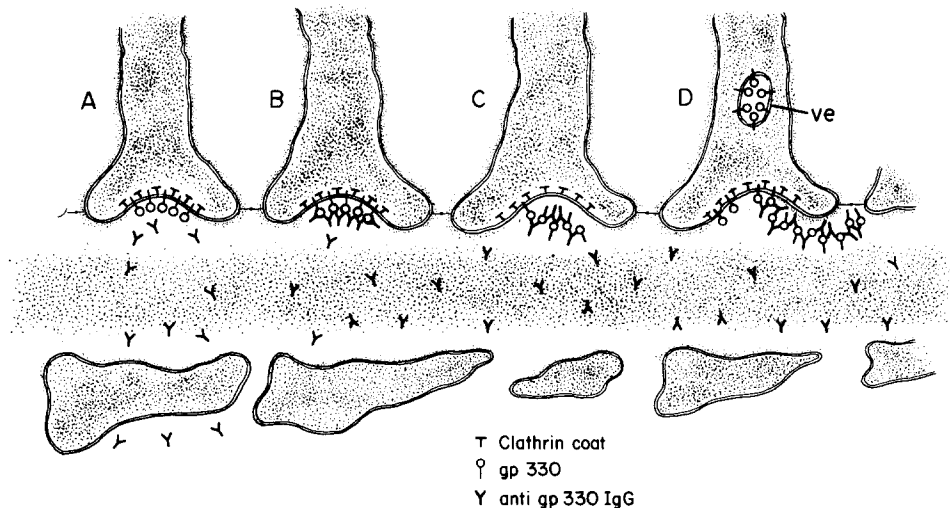
FIGURE 10. Schematic drawing of the early events in the formation of an ID in passive HN. (A) Circulating anti-gp330 IgG (Y) penetrates the GBM and approaches gp330 (♀), a resident membrane glycoprotein of clathrin-coated pits located at the base of the epithelial foot processes. (B) Anti-gp330 IgG binds to its antigen, gp330, in the coated pits forming an immune complex. (C) The initial immune complex becomes attached to the GBM as early as 15 min after injection of antibody, and is shed but remains in contact with a coated pit. (D) The ID grows in size by repeated cycles of in situ immune complex formation and shedding into the lamina rara externa until it eventually encroaches on the area of the slit diaphragm. The same result could be obtained if the ID is fixed to one site on the GBM and the foot process subsequently moves over the ID until positioned under the slit diaphragm. The continued growth of the IDs appears to require the de novo synthesis by the podocytes of new molecules of gp330 which, like other membrane glycoproteins, are assumed to be delivered via vesicles (ve) that eventually fuse with the cell membrane at the base of the foot processes.

become firmly fixed to the GBM by 15 min after antibody injection; (c) IDs remain associated with coated pits at the base of the foot processes up to 8 d after IgG injection; and (d) both the antigen (gp 330) and the antibody (anti-gp330) are present throughout the IDs at all time points.

The results of our experiments have provided new information, summarized in Fig. 10, that allow us to reconstruct the early events that occur in the

generation of IDs in passive HN as follows: Within minutes after its administration, anti-gp330 IgG penetrates the GBM and binds to its antigen, gp330, located in coated pits at the base of the foot processes. Although gp330 is a resident membrane protein of coated pits found on the epithelial cell body and on the sides and the base of the foot processes, the antibody has access only to those coated pits located on the soles of the podocyte's feet, because immunolabeling reveals that binding of IgG occurs exclusively in this site. Immediately (within 15 min) after binding, the immune complexes form morphologically recognizable IDs that become so firmly attached to the GBM that they coisolate with it. This firm attachment must involve strong, probably covalent, interactions between the GBM and the antigen or antibody because it resists high salt, detergent, collagenase, and heparinase treatments. It is disrupted only by reagents that dissociate antigen-antibody complexes. With increasing time (1 h to 8 d) after IgG injection, the IDs grow in size as a result of the continued penetration of IgG across the GBM, and they are eventually shed into the lamina rara externa (4, 5, 25, 26). It is not clear exactly when shedding occurs in relationship to attachment and growth of the IDs, but that shedding does occur is suggested by experiments on cultured glomerular epithelial cells (25, 26) and by the fact that both antigen and antibody can be demonstrated in the IDs at some distance from the epithelial cell membrane, i.e., at a distance greater than the ectodomain of the gp330 molecule would be expected to protrude from the surface of the epithelial cell membrane. The simplest explanation for the gradual increase in size of the IDs is that they grow by repeated IgG binding and shedding of the resultant immune complexes. Binding of circulating IgG directly to free antigenic sites on the shed antigen and deposition of components of complement (C3 and C5 to C9) (27–29) may also contribute to the process. For the continued growth of the IDs to take place undoubtedly requires the synthesis of new gp330 molecules and their insertion into the coated pits at the base of the foot processes. The fact that there is a lag period after the initial injection of IgG before the number of IDs increases (from ~17–90/μm GBM), together with the fact that a similar lag period was shown to occur before gp330 reappears on the surface of cultured epithelial cells after antibody-induced shedding of gp330-containing immune complexes (25), also suggests that de novo synthesis of gp330 is required. As the IDs grow in size and surface area they gradually encroach on the areas under the slit diaphragms, and their relationship to coated pits becomes less obvious. However, as determined by serial sectioning, each ID maintains contact with a coated pit on some part of its surface.

The question immediately arises as to how the IDs can maintain contact with a coated pit in the face of continual antigen shedding. This would be possible if, as suggested by the experiments of Seiler et al. (30), the epithelial cell membrane at the base of the foot processes or the entire foot process can move along the outer surface of the GBM, because as new gp330 molecules appear in coated pits along the basal epithelial membrane the antigen would likely become fixed to the IDs by binding to free sites on the deposited IgG.

The finding that the IDs become rapidly fixed to the GBM by interactions that resist hypotonic lysis and detergent extraction during GBM isolation may explain why the epithelial cells are unable to remove and dispose of the IDs.

Other filtration residues that accumulate in the lamina rara externa, such as native (anionic) ferritin (31), neutral dextrans (32), and poly-lysine-heparin complexes (33), are efficiently incorporated into the epithelium by endocytosis and disposed of in lysosomes. The fact that proteins became rapidly attached to the GBM may also explain why some exogenous proteins, especially those that are cationic in nature (34) and bind to anionic sites in the GBM, persist in glomeruli and serve as implanted antigens.

Several treatments were carried out on isolated GBMs in an attempt to gain insights into the nature of the interaction between gp330 and/or anti-gp330 and the GBM, but none released the bound ^{125}I-labeled IgG except those treatments (high or low pH) that disrupt antigen-antibody complexes. Although the mechanism(s) by which the IDs become fixed to the GBM remains unknown, several possibilities can be ruled out or considered unlikely, e.g., that it occurs by charge or hydrophobic interactions or by crosslinking to collagen or heparan sulfate, because treatment with high salt or detergent or with collagenase or heparinase failed to release bound IgG. In all likelihood the attachment is covalent, but the nature of the binding remains to be established. The GBM itself is known to be of low solubility and to be stabilized by intra- and intermolecular disulfide bridges and nonreducible bonds (35).

Several requirements for the persistence of IDs in passive HN have been established as a result of previous studies. To begin with, the presence of anti-gp330 is required. Although antibodies to other glomerular epithelial cell-surface components such as a 90–94-kD glycoprotein (11, 36) and podocalyxin (21, our unpublished results) bind to glomeruli after intravenous injection, the binding does not lead to the formation of persisting IDs. In addition, it is known that precipitating, multivalent antibodies are required because nonprecipitating antibodies (37) and mixtures of mAbs antibodies (38) were ineffective in promoting ID formation. Moreover, only immune complexes in which the antigen and antibody can condense and rearrange into larger latticed IDs persist in glomeruli and become electron-dense deposits (39). It is the inability to undergo this type of rearrangement that probably prevents ID formation in anti-GBM-type glomerulonephritis caused by binding of antibodies to antigens such as laminin (40) and heparan-sulfate proteoglycans (41) that are firmly fixed in the GBM. In the case of gp330-anti-gp330 complexes, shedding is probably a necessary prerequisite for condensation and rearrangement of immune complexes. Complement does not seem to be required for ID formation as IDs form in complement-depleted animals (27); however, complement is required for proteinuria to occur (29). Although there are circumstances where positively charged antibodies have been shown to bind to the GBM more readily than negatively charged antibodies (42, 43), our results, as well as those of Adler et al. (44), indicate that in this disease model, positively charged antibodies are not involved because the bound IgGs consisted of anionic species with acidic isoelectric points (5.0 and 6.1).

The results of our experiments raise several questions that must be answered for the molecular events leading to the formation of IDs to be fully understood and to devise appropriate strategies for preventing ID formation or their stabilization once they have formed. The most pressing is to define the nature of the interactions between gp330 and the GBM. In addition, one would like to be able

to explain why immune complexes formed in situ with gp330 are stable and grow, whereas those induced by antibodies against two other resident membrane glycoproteins of glomerular epithelial cells, i.e., podocalyxin (our unpublished data) and a 94-kD glycoprotein (11, 36), are only transient. Since both podocalyxin and the 94-kD glycoprotein are present at the surface of the glomerular endothelium as well as the epithelium, it is likely that these antibodies may be removed from the circulation before reaching the epithelium by "antibody modulation," that is, by binding of the antibody to the endothelium, followed by capping and internalization of the resultant immune complexes or their shedding into the circulation (26, 45). gp330 differs from the other two antigens mentioned in that it is exclusively an epithelial antigen that is not accessible to circulating antibodies in any organ other than the kidney, and anti-gp330 IgG persists several hours to days in the circulation (41). Finally, one would like to know to what extent formation of IDs is specific for gp330 and related to unique features of its molecular structure or functions (e.g., in endocytosis).

Summary

The nephritogenic antigen of Heymann's nephritis (HN), gp330, was previously demonstrated (4–9) to be a resident glycoprotein of coated pits in the glomerular and proximal tubule epithelium of rats, and anti-gp330 IgG given intravenously was found to form IDs in glomeruli (passive HN). The purpose of this study was to investigate the detailed events that occur in the formation of IDs in passive HN. HN was induced by the injection of either ^{125}I-labeled or unlabeled anti-gp330 IgG. At various times after injection (15 min to 8 d) the kidneys of some of the injected rats were fixed by perfusion, and the distribution of the rabbit IgG was determined by immunofluorescence and by immunoelectron microscopy. Glomeruli were isolated from the kidneys of injected rats and used for isolation of GBM fractions or for elution of the bound IgG.

At 15 min to 1 h after injection, the rabbit IgG was localized by immunocytochemistry exclusively in coated pits along the podocyte plasmalemma facing the GBM. By 1–8 d, anti-gp330 IgG was detected in larger electron-dense IDs often located under the slit diaphragms. Serial sectioning revealed that each of the IDs maintained contact with a coated pit at some level. When GBMs isolated from rats given radiolabeled anti-gp330 IgG were examined by electron microscopy, the IDs were found to remain attached to the GBMs as early as 15 min after injection and coisolated with them at all time points. By double-immunolabeling of the isolated GBMs with two sizes of gold particles, both the antigen (gp330) and the anti-gp330 IgG could be demonstrated in IDs at all time points. When the amount of radiolabeled anti-gp330 bound to GBM fractions was compared with that of isolated glomeruli, it was found that 20% of the radiolabel remained bound to the purified GBMs at 15 min after injection, and 90% at 3 d. The bound IgG was released only by treatments that disrupt antibody-antigen complexes (high and low pH), but not by the other treatments we tried (detergent, high salt, heparinase, or collagenase digestion).

When the IgG bound to glomeruli was eluted with acid citrate buffer 3 d after injection, it was found to specifically immunoprecipitate only gp330 from deter-

gent-solubilized ^{125}I-labeled kidney microvillar vesicles. By isoelectric focusing the eluate was found to be enriched in IgGs with acidic isoelectric points.

These results confirm and extend previous data (4–9) indicating that coated pits at the base of the foot processes are the sites where resident gp330 and circulating anti-gp330 meet to form the initial immune complexes. They further demonstrate that the IDs remain in contact with coated pits up to 8 d after injection. A major new finding was that, as early as 15 min after injection of anti-gp330 IgG, morphologically recognizable IDs become firmly attached to the GBM by unknown, probably covalent, interactions.

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