PRECIPITATING ANTIGEN-ANTIBODY SYSTEMS ARE REQUIRED FOR THE FORMATION OF SUBEPITHELIAL ELECTRON-DENSE IMMUNE DEPOSITS IN RAT GLOMERULI*

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In recent years the understanding of the mechanisms that lead to immune deposits in renal glomeruli has improved. The lattice of circulating antigen-antibody complexes influences the deposition of these substances in the subendothelial and mesangial areas in glomeruli. Electron-dense immune deposits evolved in subendothelial and mesangial areas when large-latticed immune complexes, defined as containing more than two antibody molecules (greater than \( Ag_2Ab_2 \)), were injected into mice (1). When small-latticed immune complexes (\( Ag_1Ab_1 \) and \( Ag_2Ab_2 \)) were injected, electron-dense deposits did not evolve (2). When the already deposited large-latticed complexes in glomeruli were exposed to large excess of antigen in vivo to convert them into small-latticed complexes, the deposits were released from glomeruli (3). Finally, even the soluble, large-latticed immune complexes had to undergo rearrangement or condensation into even larger immune complexes to persist in glomeruli and to become electron-dense deposits (4). These observations collectively indicate that the development of subendothelial and mesangial electron-dense immune deposits requires a precipitating antigen-antibody system.

The development of subepithelial antigen-antibody deposits is thought to occur principally by local immune complex formation, as reviewed by Couser and Salant (5). The recognition of fixed negative charges in the glomerular capillary wall (6–8) led to a series of experiments documenting that positively charged antigenic molecules were planted in the subepithelial area of glomeruli (9–11). When specific antibodies were injected into these animals, immune deposits and electron-dense deposits formed and persisted for days (12–14). The lattice requirements for the development of subepithelial electron-dense immune deposits has not been examined.

The experiments described in this article used cationized precipitating or nonprecipitating antigens, constructed by varying the hapten density on a carrier molecule, to show that a precipitating antigen-antibody system is required for

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the development of electron-dense immune deposits in the subepithelial area of rat glomeruli. The nonprecipitating immune complexes persisted for a short period of time by immunofluorescence microscopy comparable to the cationized antigen alone, and the presence of these complexes was not detectable by electron microscopy.

Materials and Methods

Preparation of Antigens and Antibodies. Human serum albumin (HSA) was purified by ion exchange chromatography and gel filtration. Twice recrystallized 4-fluoro-3-nitroaniline (Aldrich Chemical Co., Milwaukee, WI) was converted to 4-fluoro-3-nitrophenyl azide and conjugated to HSA by previously described methods (15) to yield nitroazidophenyl HSA (NAP-HSA). The average degree of conjugation of the hapten to HSA was determined by amino acid analysis (AAA Laboratory, Seattle, WA). Since a precipitating antigen-antibody system was achieved with NAP-HSA preparations when the average hapten density exceeded 13 (16), three conjugates were prepared, NAP, HSA, NAP, HSA as nonprecipitating antigens and NAP, HSA as a precipitating antigen. These calculations were based on the presence of 58 lysines in HSA (17). The concentrations of NAP-HSA preparations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) (18) using HSA as a standard, since the NAP group does not interfere with this reaction. The NAP-HSA preparations were protected from light with aluminum foil around the containers or columns because the aryl azide is photosensitive.

NAP-HSA preparations were cationized by previously described methods with some modifications (19). The NAP-HSA preparations were dialyzed into 0.2 M phosphate buffer, pH 6.0. Ethylene diamine (340 \mu l) was added to 7.0 ml of the same phosphate buffer with stirring and the pH was adjusted to 6.0 with 12 M HCl, and the volume was brought to 10 ml with the phosphate buffer. Then 20 mg of protein in 3.0 ml was added, followed with 400 mg of EDAC [1-ethyl-3-(3-dimethylamino-propyl) carbodiimide HCl; Sigma Chemical Co., St., Louis, MO] and the mixture incubated at room temperature for 30 min. The reaction mixture was then extensively dialyzed against 0.2 M sodium borate, 0.15 M NaCl, pH 8.0, buffer. Monomeric, cationized antigen was obtained by gel filtration over Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ). The cationized antigens were designated NAP, HSA, NAP, HSA, and NAP, HSA. The flat bed Pharmacia isoelectric focusing system (Pharmacia Fine Chemicals) was used to characterize the degree of cationization of antigens, as previously described (19).

Antibodies to the NAP haptenic group were obtained by immunizing rabbits with NAP-keyhole limpet hemocyanin. The antiserum was heated to 56°C for 30 min to inactivate complement, and antibodies to NAP (antiNAP) were isolated with affinity chromatography as previously described (20), using NAP-HSA affinity columns. Antibodies to HSA were obtained by immunizing rabbits with HSA in complete Freund’s adjuvant. The specific antibodies were isolated by affinity chromatography as previously described (20). The antibody preparations were gel filtered on Sephadex G-200 columns (Pharmacia Fine Chemicals) to obtain monomeric preparations.

Double immunodiffusion was carried out, as described by Ouchterlony (21), to determine the precipitability of the antigen-antibody systems. Agarose IEF (Pharmacia Fine Chemicals) was used to construct the gels to permit free diffusion of the cationic antigens.

Radioiodination of antigens or antibodies with \(^{125}\text{I}\) or \(^{131}\text{I}\) was carried out by the iodine monochloride method (22), yielding specific activities of 1.5–2.0 \(\mu\)Ci/mg of protein. Sucrose density gradient ultracentrifugation for characterization of the lattice of immune

\(^{1}\) Abbreviations used in this paper: antiHSA, rabbit antibodies to human serum albumin; antiNAP, antibodies to the nitroazidophenyl haptenic group; ED, ethylene diamine treated for cationization; EDAC, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide HCl; HSA, human serum albumin; NAP, 2-nitro-4-azidophenyl; NAP-HSA, nitroazidophenyl human serum albumin; the subscript after NAP designates the average number of the haptenic groups in HSA.
complexes was conducted on 10–30% linear sucrose gradients as previously described (20).

Animal Experiments. Lewis or Sprague-Dawley rats weighing 350–450 g were used. Anesthesia was achieved with intramuscular 0.2 ml of 1:5 dilution of Inovar-vet (McNeil Laboratories, Inc., Fort Washington, PA). All injections of antigen were given directly into the left renal artery. For this purpose a midline abdominal incision was made with rats under anesthesia. The left renal artery was then exposed, and 0.5 ml of borate buffer was used to flush the left kidney through a 25-gauge needle inserted into the renal artery, followed by antigen preparations in 0.5 ml volume. Blood flow was then immediately restored to the kidney. The total ischemia time was 1–2 min. For immune complex formation in kidneys, 1 mg of antiNAP or 1 mg of antiHSA was given intravenously 10 min after an antigen was infused into the left renal artery.

Renal biopsies were obtained at the designated times, cutting a wedge-shaped piece from the renal cortex, approximately 3 mm × 3 mm and 3–4-mm deep. The specimens during the first hour were obtained while the rats were under initial anesthesia. The later specimens were obtained by open renal biopsy under anesthesia or after the rats were sacrificed by an overdose of the anesthetic. The obtained tissue was divided for immunofluorescence and electron microscopy and processed immediately.

The tissue for immunofluorescence microscopy was embedded in Tissue-Tek II O.C.T. Compound (Ames Co., Elkhart, IN), quick frozen with CO₂, and stored at −20°C until studied further. 6-μm sections were cut, washed, and stained with specific fluoresceinated antibodies as previously described (1). Specific fluoresceinated goat antibodies to rabbit IgG, human serum albumin, rat IgG, and rat C3 were employed (Kallestad Laboratories, Chaska, MN). The presence of NAP antigenic determinants was detected by indirect immunofluorescence, using first unconjugated, purified rabbit antibodies to NAP and followed after washing with fluoresceinated goat antibodies to rabbit IgG.

The tissues for electron microscopy were fixed in glutaraldehyde buffered with sodium cacodylate. They were then postfixed in 1% buffered osmium tetraoxide and embedded in epoxy resin. Thin sections were cut on the LKB III Ultratome, stained for 2 h with uranyl acetate, then lead citrate for 5 min. The sections were then examined with transmission electron microscope (Type 801 AEI Scientific Apparatus Division, Harlow, Essex, England). A minimum of eight glomeruli were examined in each specimen.

Results

Characterization of NAP-HSA Preparations. By isoelectric focusing the cationized preparations showed pI of 8.5–10.0 (Fig. 1). Double immunodiffusion of all three preparations showed precipitin lines with antibodies to HSA. In contrast, precipitin lines were present only with the multivalent preparation (NAP₁₉.₇-HSA₁ₓ) when antibody to the NAP haptenic group was used (Fig. 2). The oligovalent antigen preparations (NAP₃₃₁.₄-HSA₁ₓ and NAP₁₁₄·HSA₁ₓ) inhibited the precipitin line with NAP₁₉.₇·HSA₁ₓ, indicating that the oligovalent antigen preparations combined with the antibody, but were unable to form precipitates. The cationized and noncationized preparations showed no differences by double immunodiffusion.

To ascertain that the antigen with lowest valence (NAP₃₃₁.₄·HSA₁ₓ) was able to form soluble immune complexes, an aliquot of this preparation was labeled with ^{131}I and immune complexes were made with antiNAP in antibody excess, and analyzed on sucrose density gradient. In antibody excess 25% of the radiolabeled material was not combined with antibodies, indicating that not all molecules carried the NAP determinants. The principal species of formed antigen-antibody complexes had a sedimentation constant of 8.1 Svedberg units and therefore must have consisted of Ag₁Ab₁ lattice (23). Complexes with sedimentation
SUBEPITHELIAL IMMUNE DEPOSITS IN GLOMERULI

FIGURE 1. Isoelectric focusing patterns of human serum albumin and cationized precipitating and nonprecipitating NAP-HSA preparations. Lane a shows the pattern for NAP$_{31}$-HSA$_{ED}$, the majority of which has a pH $>$8.65. In lane b, NAP$_{19.7}$-HSA$_{ED}$ focuses predominantly in a pH range $>$8.65; the material present at the lower pH (7.35) is at the point of application, and a result of poor penetration of the agarose by the highly substituted NAP-HSA. Lane c shows the pH for unaltered HSA. Lane d shows the standards.

FIGURE 2. Photograph of double immunodiffusion in agarose IEF of cationized and noncationized NAP-HSA preparations against antiNAP (center well A). Peripheral wells contain the following: well 1, empty; well 2, NAP$_{31}$-HSA$_{ED}$; well 3, NAP$_{31}$-HSA$_{ED}$; well 4, NAP$_{31}$-HSA; well 5, NAP$_{19.7}$-HSA; well 6, empty. Precipitin lines form between antiNAP and both the cationized and noncationized NAP$_{19.7}$-HSA preparations. The NAP$_{31}$-HSA preparations failed to form precipitin lines with antiNAP, but inhibited the precipitin lines formed between antiNAP and the NAP$_{19.7}$-HSA preparations.
constant of 11.7 \((\text{Ag}_2\text{Ab})\) and even larger complexes were present in small amounts (Fig. 3). The NAP-HSA\text{ED} preparations with higher hapten density formed a higher proportion of large-latticed complexes at 5 times antigen excess than the NAP\text{3.1}-HSA\text{ED} in antibody or antigen excess (data not shown).

**Glomerular Localization of Antigen Alone.** The localization and persistence in glomeruli of the cationized NAP-HSA preparations alone were defined. After initial trials with several dosages, 600 \(\mu\)g of NAP\text{3.1}-HSA\text{ED} and NAP\text{11.4}-HSA\text{ED} and 400 \(\mu\)g NAP\text{19.7}-HSA\text{ED} were chosen as the dosages to be examined in detail, to assure maximum glomerular binding of the antigen. A higher dose was chosen for the nonprecipitating NAP-HSA preparations because not all HSA molecules carried the NAP haptenic groups, as described above. Separate groups of rats were injected with each antigen preparation. The left kidney of each rat was biopsied at 1 min after injection and then at additional times so that at least two specimens were obtained from different rats at 10, 60 min, and 4, 8, 24, and 96 h after the injection of each antigen preparation. Since sequential biopsies were obtained in all rats multiple biopsies were not obtained at each time point. The presence of the antigens alone in glomeruli was examined by indirect immunofluorescence staining for the NAP haptenic groups and by direct immunofluorescence staining for HSA as the carrier molecule. With staining for the NAP haptenic group the antigen with the lowest hapten density (NAP\text{3.1}-HSA\text{ED}) localized and persisted in glomeruli for the shortest period of time, being 4+ at 1 min and 2+ at 10 min after antigen injection (Table I). When stained for HSA, the carrier molecule, this antigen persisted in glomeruli through 4 h. With NAP\text{11.4}-HSA\text{ED} and NAP\text{19.7}-HSA\text{ED} the immunofluorescent staining for NAP haptenic groups and for the HSA gave parallel results. By 8 h these antigens had nearly completely disappeared from glomeruli and were absent by 24 h (Table I). With the NAP\text{3.1} HSA in 300-\(\mu\)g, 400-\(\mu\)g, and 1,000-\(\mu\)g doses the results were similar to those indicated for the 600-\(\mu\)g dose in Table I.

On the basis of these data 10 min after the infusion of the antigens into the

![Figure 3](image-url)

**Figure 3.** Sucrose density gradient ultracentrifugation pattern of \(^{131}\text{I-NAP}_{11.4}\cdot\text{HSA}_{\text{ED}}\)
anti-NAP immune complexes made in antibody excess. \(\sim 25\%\) of free antigen is present. The majority of the immune complexes sedimented in the 8.1S peak, indicating \(\text{Ag}_1\text{Ab}_1\) complexes. Smaller amounts of 11.7S (\(\text{Ag}_2\text{Ab}_2\)) and 17.1S complexes were also present.
left renal artery was chosen as the time for intravenous administration of antibodies to examine for formation of immune complexes in glomeruli.

**Formation and Persistence of Immune Complexes in Glomeruli.** Following the injection of 600 μg of cationized, nonprecipitating antigens or 400 μg of cationized, precipitating antigen into the left renal artery, 1 mg of purified rabbit antiNAP was injected intravenously. Separate groups of rats were used with each antigen preparation. The left kidney of each rat was biopsied at 12 min (2 min after antibody injection) and at additional times so that single or multiple biopsy specimens were obtained from different rats at staggered time points at 40, 60 min and 4, 8, 24, and 96 h. The times of biopsies were staggered so that tissue was obtained from each rat early and late in the course of the experiment. The rats given the precipitating antigen (NAP19.7-HSAED) and antiNAP showed presence of the carrier molecule (HSA) and the antibodies (rabbit IgG) from 2 min after infusion of antibody to 96 h by immunofluorescence microscopy (Table II, Fig. 4). In contrast, the groups of rats given nonprecipitating antigens (NAP3.1-HSAED or NAP11.4-HSAED) and antiNAP showed the presence of the antigen molecules and antibodies from 2 min after infusion of antibody to 4 h and only small amounts of rabbit IgG at 8 h and were completely negative by 24 h and later (Table II, Fig. 4).

To determine that the same nonprecipitating antigens (NAP3.1-HSAED and NAP11.4-HSAED) with antiNAP were able to form persisting immune deposits in glomeruli with antibodies to the carrier molecule, purified rabbit antibodies to human serum albumin (antiHSA) were given in the same dose after the antigen injection to other rats and biopsied by a similar schedule as described above. In these rats the antigen molecules and antibodies were present in glomeruli by immunofluorescence microscopy from 2 min through 96 h after antibody administration (Table II).

The injection of antiNAP or antiHSA alone into rats without prior antigen administration caused no glomerular deposits by immunofluorescence microscopy with antibodies to rabbit IgG.

All renal biopsies were also stained with goat antibodies to rat IgG and no
TABLE II

Glomerular Presence of Rabbit IgG and HSA After Injection of Nonprecipitating or Precipitating Antigens, Followed by Administration of Antibodies*

| Time of biopsy | Rats given antiNAP after antigen | Rats given antiHSA after antigen |
|---------------|----------------------------------|---------------------------------|
|               | NAP$_{3.1}$·HSA$_{ED}$          | NAP$_{11.4}$·HSA$_{ED}$         | NAP$_{19.7}$·HSA$_{ED}$         | NAP$_{3.1}$·HSA$_{ED}$          | NAP$_{11.4}$·HSA$_{ED}$         |
|               | Ab | Ag | Ab | Ag | Ab | Ag | Ab | Ag | Ab | Ag |
| 12 min        | 4+ | 4+ | 3+ | 3+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| 40 min        | 4+ | 4+ | 3+ | 3+ | 3.5+ | 4+ | 4+ | 4+ | 3.5+ | 3+ |
| 60 min        | 3+ | 3+ | 2+ | 3+ | 3+ | 3+ | 4+ | 4+ | 3+ | 3+ |
| 4 h           | 2.5+ | 2+ | 2.5+ | 1+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ |
| 8 h           | ± | 0 | 1+ | 0 | 3.5+ | 3+ | 4+ | 2.5+ | 3+ | 2+ |
| 24 h          | 0 | 0 | 0 | 0 | 3+ | 3+ | 3+ | 2+ | 2.5+ | 2+ |
| 96 h          | 0 | 0 | 0 | 0 | 3+ | 2+ | 3+ | 2+ | 2.5+ | 2+ |

* All tissue specimens were stained with fluoresceinated goat anti-rabbit IgG; the results are given in columns designated Ab, and stained with fluoresceinated goat anti-HSA; the results are given in columns designated Ag.

‡ Rats were given 600 µg of the antigen into left renal artery and 10 min later 1 mg of antibodies intravenously.

§ Rats were given 400 µg of the antigen into left renal artery and 10 min later 1 mg of antibodies intravenously.

deposits were found at any time point in the described experiments. Similar staining was also performed with goat antibodies to rat C3, only the specimens with persistent presence of antigen and antibody at 96 h showed trace amounts of rat C3.

Transmission electron microscopy was carried out on selected specimens of renal tissues to determine the presence and location of electron-dense deposits. In rats given the nonprecipitating antigens (NAP$_{3.1}$·HSA$_{ED}$ and NAP$_{11.4}$·HSA$_{ED}$) and antiNAP, electron-dense deposits could not be found at 40 min or 60 min (Fig. 5), even though the antigen and antibodies were present by immunofluorescence microscopy (Table II). In contrast, in glomeruli of rats given the precipitating antigen (NAP$_{19.7}$·HSA$_{ED}$) and antiNAP, electron-dense deposits were found as early as the 40-min time point, at which time the loose deposits were largely in the subendothelial and mesangial areas, with sparse subepithelial deposits. By 4 h, the electron-dense deposits were principally in the subepithelial area, with occasional mesangial deposits. By 24 and 96 h, only subepithelial electron-dense deposits were present (Figs. 5 and 6). Also in rats given the nonprecipitating NAP·HSA preparations, followed by antibodies to HSA, subepithelial deposits were present at 24 h and 96 h. These deposits were identical to the deposits found in rats given the precipitating NAP·HSA preparation and antiNAP at the same time points.

Discussion

The presented data indicate that precipitating antigen-antibody systems are essential for the persistence of immune complexes by immunofluorescence microscopy and the formation of electron-dense immune deposits in the subepithelial area of glomeruli. With nonprecipitating antigen-antibody systems immune
complexes formed in glomeruli as determined by immunofluorescence, but these complexes were present only transiently, comparable to the antigen molecules alone, and electron-dense deposits did not evolve in glomeruli with these preparations.

The infusion of antigens alone demonstrated that the cationized NAP·HSA preparations were readily planted in glomeruli due to interaction with the fixed negative charges, that have been demonstrated in the lamina rara interna and in the lamina rara externa of the glomerular basement membrane (6). The presence of antigen molecules was demonstrated by immunofluorescence microscopy with antibodies to the hapten and with antibodies to the carrier molecule. By staining with antibodies to the hapten, the NAP\textsubscript{3,1}·HSA preparation was present in
Figure 5. Electron photomicrographs of the glomerular capillary basement membrane in rats injected with cationized NAP-HSA preparations into the left renal artery, followed by antiNAP intravenously at 10 min. In panel A the rat received NAP₁₁.₁-HSAₑᵈ followed by antiNAP and renal biopsy was taken at 60 min. No electron-dense deposits are seen. In panel B the rat received NAP₁₉.₇-HSAₑᵈ followed by antiNAP and renal biopsy was taken at 60 min. Prominent, loose subendothelial deposits (arrows) are seen, with loss of endothelial cell fenestrae. In panel C the rat received NAP₉₉.₇-HSAₑᵈ followed by antiNAP, and biopsy was taken at 96 h. Prominent electron dense deposits (arrows) are seen in the subepithelial region. US, urinary space; EC, endothelial cell; CL, capillary lumen; PMN, polymorphonuclear leukocyte. A–C, × 19,000.

glomeruli at 1 min and 10 min after injection into the renal artery. The staining of the tissues with antibodies to carrier molecules, however, disclosed that the injected material was present longer and became undetectable by 8 h after injection. The difficulty in detection of the antigen molecules with antibodies to NAP was attributed to the low valence of the antigen. With higher hapten density on HSA (NAP₁₁.₁-HSAₑᵈ and NAP₁₉.₇-HSAₑᵈ), the presence of the injected molecules could be detected in comparable manner with antibodies to the hapten or with antibodies to the carrier molecule. Because of this apparently short presence of the haptenic determinants with NAP₃.₁-HSAₑᵈ, 10 min was chosen as the time for injection of the antibodies to NAP determinants in all experiments.
The persistence of the cationized antigens alone in glomeruli was comparable to findings by previous investigators; for example Oite et al. (13) determined that the half-life of cationized human IgG was 4.2 h in glomeruli of rats.

The conclusion that immune complexes were actually formed in glomeruli when NAP$_{10.7}$-HSA$_{ED}$ or NAP$_{11.4}$-HSA$_{ED}$ were injected and followed with antibodies to NAP, was based on several observations. First, even though these antigens were nonprecipitating, soluble immune complexes were formed when radiolabeled antigens were mixed with antibodies and analyzed on sucrose density gradients. In the NAP$_{3.1}$-HSA$_{ED}$ preparations 25% of the molecules did not react with antiNAP in antibody excess, either because this proportion of HSA molecules was not conjugated with the hapten or because the formed complexes dissociated during the separation on sucrose density gradient due to low avidity. It is unlikely that from this preparation the cationized HSA molecules preferentially interacted with the fixed negative charges in glomeruli. Second, after the administration of antibodies to NAP, both the antibody and antigen molecules were detected by immunofluorescence microscopy for comparable duration. Finally, the injection of NAP$_{11.4}$-HSA$_{ED}$ followed by antiNAP at the same time intervals showed comparable results (Table II). This average number of hapten groups per carrier molecule was chosen to bring the antigen close to a precipitating system with antibodies to the hapten, since previous studies showed that when the average number of NAP groups per carrier molecule was 12.9 or higher, a precipitating antigen-antibody system was generated (16).

The administration of antibodies to the carrier molecule after the injection of the NAP$_{3.1}$-HSA$_{ED}$ or NAP$_{11.4}$-HSA$_{ED}$ showed that these molecules were planted in a manner comparable with the precipitating NAP$_{10.7}$-HSA$_{ED}$ system since with antibodies to HSA the deposits persisted by immunofluorescence microscopy and
electron-dense deposits were formed in subepithelial areas of glomeruli.

The persistence of a precipitating antigen-antibody system in glomeruli and the formation of subepithelial electron-dense deposits was demonstrated with the precipitating NAP$_{19.7}$-HSA$_{ED}$ and antiNAP system and by injection of antibodies to the carrier protein with the nonprecipitating hapten-carrier conjugates. The sequence of electron-dense deposit formation was studied in detail with the NAP$_{19.7}$-HSA$_{ED}$ and antiNAP system. Already at 40 min electron-dense deposits were noted in the subendothelial and mesangial areas, but these deposits were relatively loose in character. Presumably these subendothelial deposits were formed when injected antibodies combined with the cationized antigen that had interacted with the fixed negative charges in the lamina rara interna. At 40 min only sparse subepithelial deposits were found. By 4 h the electron-dense deposits were principally in the subepithelial area and at 24 and 96 h these deposits were only in the subepithelial area. This sequence of development of electron-dense deposits indicates that if similar deposits had formed with the nonprecipitating antigen-antibody systems, the deposits should have been detected. The build-up of subepithelial immune deposits may involve two mechanisms, as discussed by Vogt et al. (14). First, antigens of appropriate molecular size may plant both in the lamina rara externa and lamina rara interna and then form immune complexes in both locations with antibodies reaching these locations from the circulation. Second, immune deposits may initially form in the subendothelial area and then dissociate into smaller complexes or even free antibodies and antigens and these small complexes or free antibodies may pass through the lamina densa to form immune complexes in the subepithelial area and condense into immune deposits. These two possible mechanisms are consistent with the presented results and the available information does not distinguish between these alternatives. The observed sequence of development of electron-dense deposits with the precipitating antigen-antibody systems was comparable to the sequence with cationized ferritin and antibodies to ferritin (14).

The mechanism by which cationized antigen molecules are removed from the subepithelial areas of glomeruli is not known at this time. Of interest is that small antigen-antibody complexes are removed from this area in a similar time period, and therefore possibly by the same unknown mechanism.

In a previous investigation we concluded that circulating immune complexes must undergo condensation or rearrangement after the initial attachment to glomeruli to form electron-dense deposits in subendothelial or mesangial areas of glomeruli in mice (4). Thus, in conjunction with the data presented here the conclusion can be reached that the formation of electron-dense immune deposits in the glomeruli, i.e. in subendothelial, mesangial, or subepithelial area, requires precipitating antigen-antibody systems. This information should be useful in the identification of antigen-antibody systems that cause glomerulonephritis in humans associated with the presence of electron-dense immune deposits. The relatively transient presence of immune complexes that can not form precipitates is detectable by immunofluorescence microscopy by staining for antibodies or for antigens, but can not be recognized by routine electron microscopy. The ability of these transient, smaller immune complexes to induce inflammation or
to cause damage by complement activation in the subepithelial area have not been examined.

Summary

This study was conducted to determine whether multivalent, precipitating antigens are required for formation of subepithelial electron-dense immune deposits in glomeruli.

2-nitro-4-azidophenyl (NAP) was conjugated with variable density to human serum albumin (HSA) to yield nonprecipitating (NAP <sub>1.1</sub> HSA and NAP <sub>1.4</sub> HSA) and precipitating (NAP <sub>1.7</sub> HSA) antigens with antibodies to the hapten. These antigen preparations were cationized with ethylene diamine to enhance deposition in renal glomeruli due to interaction with the fixed negative charges in the glomerular capillary wall. Following injection into the left renal artery of rats these antigens alone persisted in the glomeruli for a relatively short time by immunofluorescence microscopy. When antibodies to NAP were injected intravenously after the antigen injection, the nonprecipitating antigens and antibodies were detectable in the glomeruli by immunofluorescence microscopy up to 8 h, comparable to antigen alone. Electron-dense deposits were not formed in these glomeruli. In contrast, when the precipitating antigen was injected and followed by antibodies to the hapten, antigen and antibody were detected by immunofluorescence microscopy through 96 h. In these specimens electron-dense deposits were present from 40 min through 96 h and after 24 h the deposits were present only in the subepithelial area. The same results were obtained when the nonprecipitating hapten-carrier conjugates were followed with antibodies to the carrier molecule. These data indicate that the persistence of immune deposits by immunofluorescence microscopy and the formation of electron-dense deposits in the subepithelial area require a precipitating antigen-antibody system.

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