

# REARRANGEMENT OF IMMUNE COMPLEXES IN GLOMERULI LEADS TO PERSISTENCE AND DEVELOPMENT OF ELECTRON-DENSE DEPOSITS\*

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The presence of antigen-antibody complexes as electron-dense deposits in glomeruli is well established in experimental models and in human glomerulonephritis. The principal locations of these deposits are the subendothelial area, the mesangial matrix, and the subepithelial area. The subendothelial and mesangial immune deposits are thought to arise from circulating immune complexes (1), whereas several lines of evidence indicate that subepithelial immune deposits are not deposited from circulation but are locally formed (2). When immune complexes with lattice larger than  $Ag_2Ab_2$  were injected intravenously, electron-dense deposits evolved in endothelial fenestrae, the subendothelial area adjacent to the mesangium, and subsequently in the mesangial matrix (1). The size of these deposits varied from 50 to 750 nm in diameter, which was larger than the injected complexes that ranged up to 22 Svedberg U in sedimentation characteristics. The development of electron-dense deposits from circulating immune complexes suggests that after the initial deposition in glomeruli these complexes undergo rearrangement to larger complexes that become identifiable by transmission electron microscopy.

To provide experimental proof for local rearrangement of immune complexes in the formation of electron-dense deposits, covalently cross-linked immune complexes were prepared which possessed a fixed lattice and could not reequilibrate. Aliquots of the same immune complexes, non-cross-linked and with comparable lattice, served as control preparations. The cross-linked and non-cross-linked immune complexes initially showed comparable deposition but only the non-cross-linked complexes evolved into electron-dense deposits that persisted in glomeruli.

## Materials and Methods

*Preparation of Antigens, Antibodies, and Immune Complexes.* A previously described system was used, consisting of multivalent 2-nitro-4-azidophenyl-human serum albumin (NAP-HSA)<sup>1</sup> as the antigen and specific antibodies to the haptenic group. The azide group on the hapten served to establish a covalent bond in the antibody-combining site (3). Briefly, monomeric NAP-HSA was prepared with the average of 21.6 or 19 NAP groups on each HSA molecule. For

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<sup>1</sup> *Abbreviations used in this paper:* Anti-HSA, antibodies to human serum albumin; anti-NAP, rabbit antibodies to the nitroazidophenyl haptenic group; HSA, human serum albumin; NAP-HSA, 2-nitro-4-azidophenyl-human serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

some experiments the NAP<sub>19</sub>·HSA was trace labeled with <sup>125</sup>I by the iodine monochloride method as previously described (4). Antibodies to the NAP haptenic group (anti-NAP) were raised in rabbits by immunization with NAP·keyhole limpet hemocyanin, isolated with affinity chromatography, labeled with <sup>125</sup>I, and rendered monomeric with gel filtration (3).

Soluble antigen-antibody complexes with NAP·HSA and anti-NAP were made at five times antigen excess. These preparations were divided into two aliquots. One half was retained without irradiation as the non-cross-linked complexes. The other half was irradiated for 2 h with light (400–500 nm wavelength) to convert the azide group on the hapten to a nitrene group that, by insertion into a carbon-hydrogen bond, established a covalent bond between the antigen and the antibody (3).

The cross-linked antigen-antibody preparation with NAP<sub>21.6</sub>·HSA was used without further modification. Since this preparation showed 24.6% of free antibody on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the subsequent preparation was further modified to decrease the amount of antibodies not covalently cross-linked with antigen. First, the immune complex preparation with NAP<sub>19</sub>·HSA, containing 50 mg of antibodies, was irradiated as described above and then gel filtered on a Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column in borate buffer (0.2 M borate, 0.15 M NaCl, pH 8.0) to remove excess antigen. The material excluded from this column was pooled, concentrated, and then passed over an affinity chromatography column consisting of NAP·HSA conjugated to agarose. This was carried out to remove any NAP·HSA-anti-NAP complexes that would bind to the solid phase NAP·HSA antigen by free antibody-combining sites or by combining sites that were non-cross-linked and would equilibrate with the excess solid-phase antigen. The fall-through material was pooled for subsequent steps. To further diminish potentially available antibody-combining sites, NAP·lysine (3) was added in molar quantity equal to the molar amount of total antibody present in the preparation. After overnight incubation at 4°C the mixture was irradiated for 2 h and unbound NAP·lysine was removed by dialysis. Then, 4 mg of ovalbumin (Sigma Chemical Co., St. Louis, MO) was added per milligram of antibody to decrease anticipated losses of the complexes during subsequent processing, and the solution was concentrated by ultrafiltration using a PM-10 membrane (Amicon Corp., Lexington, MA) to 3.4 mg of antibody in immune complexes per milliliter of solution. Small losses occurred at each preparation step so that the final cross-linked complexes contained 60% of the starting antibodies.

*Characterization of the Prepared Antigen-Antibody Complexes.* The size distribution of the cross-linked and non-cross-linked immune complexes was assessed on 10–30% linear sucrose density gradient ultracentrifugation (5). The degree of covalent cross-linking of the preparations used in animal experiments was assessed by SDS-PAGE in the presence of 0.5 M urea (3).

The disappearance of cross-linked and non-cross-linked immune complexes from circulation of mice was determined by previously described methods (3). Briefly, 1.7 mg of antibody in immune complexes in 0.5 ml of borate buffer were injected into three C57BL/6J mice by the tail vein. Blood samples (20  $\mu$ l) were obtained from the retro-orbital plexus at 2, 4, 6, 10, 15, 30, and 60 min and then 10 additional times within the 96 h period; samples from each group were obtained at the same time. The radioactivity precipitable with cold 10% trichloroacetic acid was counted. The 0 time value was extrapolated from the first three values and the fraction remaining in circulation and the means and standard deviations were calculated for each time point, plotted, and analyzed by graphic peeling (6).

*Renal Localization of Immune Complexes.* Female C57BL/6J mice, weighing ~20 g were used. Immune complexes of each preparation were injected in 0.5 ml volume containing 1.7 mg of antibody in immune complexes for each dose. The injections were given at time 0, 20, and 40 min, using one or two mice for each time point. Three injections were given for two reasons. First, high concentrations of antibodies were avoided to minimize losses. Second, multiple injections would prolong the exposure of kidneys to immune complexes, since as a function of the NAP groups on HSA, the half-lives of the antigen alone and of immune complexes containing the antigen were shortened (3, 4). Mice were sacrificed at the stated times and kidneys were removed and processed for immunofluorescence and electron microscopy as previously described (1). The following specific, fluoresceinated antibodies were used to examine the renal tissues: goat antibodies to rabbit IgG (Kallestad Laboratories Inc., Chaska, MN),

goat antibodies to HSA (anti-HSA) (Kallestad Laboratories, Inc.) to stain for the presence of the carrier molecule in NAP·HSA, and goat antibodies to mouse C3 (Cappel Laboratories, Cochranville, PA). The intensity of fluorescence in glomeruli was graded 0-4+, in peritubular capillaries 0-2+, and  $\pm$  to indicate a trace of fluorescence. A minimum of eight glomeruli was examined by electron microscopy in each specimen. The presence of electron-dense deposits was graded as ++ (extensive), + (few),  $\pm$  (occasional), and 0 (none).

## Results

*Characteristics of the Immune Complex.* Because the initial preparation of photoactivated immune complexes with NAP<sub>21.6</sub>·HSA contained 25.2% of antibodies that were not covalently cross-linked, additional steps were taken to decrease the antibody-combining sites potentially available for rearrangement of the lattice. With the photoactivated NAP<sub>19</sub>·HSA-anti-NAP complexes, gel filtration and affinity chromatography with agarose-antigen columns were used sequentially for this purpose. As a final step, free hapten was added and photoactivation was carried out to covalently cross-link the hapten to any antibody-combining sites accessible to the hapten. The cross-linked and non-cross-linked final preparations of immune complexes used for the glomerular localization studies had comparable lattices as determined by sucrose density gradient ultracentrifugation (Fig. 1). Both small-latticed (Ag<sub>1</sub>Ab<sub>1</sub> and Ag<sub>2</sub>Ab<sub>2</sub>) and large-latticed (greater than Ag<sub>2</sub>Ab<sub>2</sub>) complexes were present, comparable to the immune complexes made with HSA and anti-HSA in earlier studies (7). By SDS-PAGE analysis the nonirradiated complexes were dissociated into free antibody and antigen, whereas in the final preparation of the cross-linked immune complexes with NAP<sub>19</sub>·HSA, only 9.5% of antibodies were not covalently cross-linked (Fig. 2).

Since the NAP groups present on HSA enhanced the clearance of immune complexes containing NAP·HSA (3), the clearance of the immune complexes used for glomerular localization was examined. When single doses of <sup>131</sup>I-NAP<sub>19</sub>·HSA and <sup>125</sup>I-anti-NAP complexes, prepared identically to the non-cross-linked complexes used for glomerular localization, were injected into mice, the antigen and antibody were

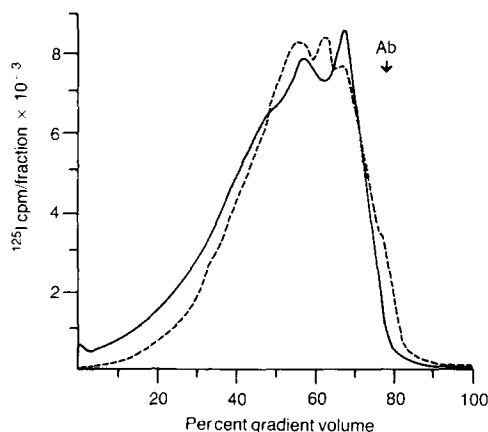


FIG. 1. Sucrose density gradient ultracentrifugation patterns of non-cross-linked and cross-linked NAP<sub>19</sub>·HSA-<sup>125</sup>I-anti-NAP complexes used for experiments in mice. 10-30% sucrose was used for linear gradients; the top of the gradient is represented by 100% of gradient volume. The non-cross-linked (---) and the cross-linked (—) complexes had comparable lattice as shown by the distribution of radiolabeled antibodies in the complexes. The position of sedimentation of antibody alone is marked with an arrow.

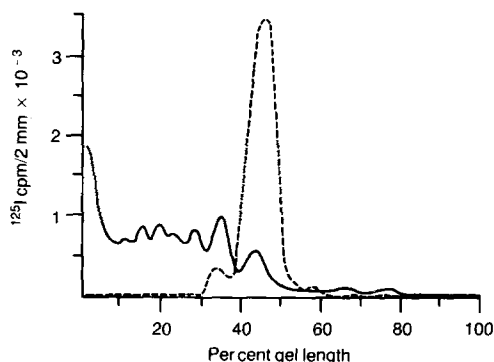


FIG. 2. SDS-PAGE patterns of non-cross-linked and cross-linked  $\text{NAP}_{19}\cdot\text{HSA}\cdot^{125}\text{I}\text{-anti-NAP}$  complexes used for experiments in mice. The non-cross-linked complexes (---) were dissociated into free radiolabeled antibody and unlabeled antigen. From the cross-linked complexes (—), 9.5% of the radiolabeled antibody was dissociated and migrated in the same area as the antibodies dissociated from non-cross-linked complexes.

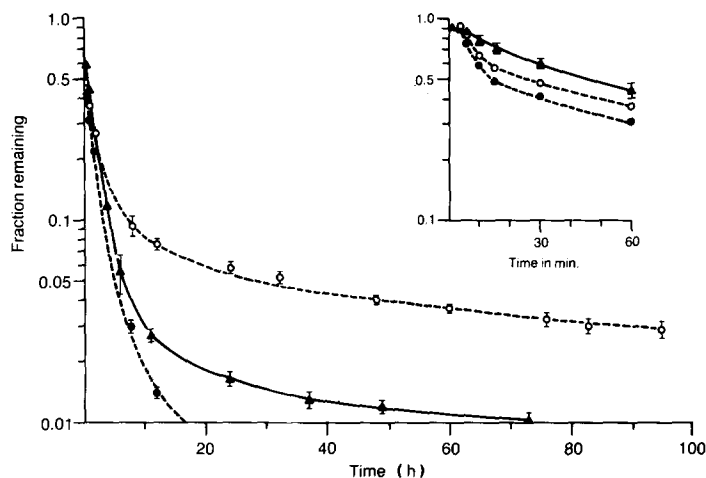


FIG. 3. Curves for the removal of non-cross-linked  $^{131}\text{I}\text{-NAP}_{19}\cdot\text{HSA}\cdot^{125}\text{I}\text{-anti-NAP}$  and cross-linked  $\text{NAP}_{19}\cdot\text{HSA}\cdot^{125}\text{I}\text{-anti-NAP}$  complexes from the circulation of mice. The mean  $\pm 1$  SD is plotted for each experimental point. The  $^{131}\text{I}\text{-NAP}_{19}\cdot\text{HSA}$  ( $\bullet$ ) and the  $^{125}\text{I}\text{-anti-NAP}$  ( $\circ$ ) in non-cross-linked complexes were initially removed together as seen in the insert for the 60 min time period; after 4 h the antigen removal continued at a fast rate, leaving the dissociated antibody in circulation. The  $^{125}\text{I}\text{-anti-NAP}$  ( $\blacktriangle$ ) in cross-linked complexes was initially removed comparably to the non-cross-linked complexes, and  $<2\%$  of the antibody in the cross-linked complexes showed a slow clearance.

initially removed in comparable manner. After 4 h, however, the antigen removal was more rapid than antibody removal (Fig. 3). After  $\sim 20$  h essentially only the  $^{125}\text{I}$ -antibody remained in circulation, and it was degraded with a half-life of 94 h. The intercept of this exponential component (5.7%) suggests that  $\sim 10\%$  of the antibodies were dissociated from the immune complexes, considering the previously examined clearance of anti-NAP alone (3). These observations indicated that the immune complexes were dissociated in circulation due to the previously known rapid clearance of  $\text{NAP}\cdot\text{HSA}$  with more than seven NAP groups (4). In contrast, with purified, covalently cross-linked  $\text{NAP}_{19}\cdot\text{HSA}\cdot^{125}\text{I}\text{-anti-NAP}$  complexes, the clearance of the  $^{125}\text{I}\text{-anti-NAP}$  was initially comparable to the clearance of antibody in non-cross-

linked complexes (Fig. 3). <2% of the radioactivity had a slow exponential component that paralleled the clearance of the antibody dissociated from complexes that were non-cross-linked. As previously shown, in cross-linked immune complexes the removal of antigen and antibody was parallel (3) and therefore in the present study dual labeling of the cross-linked complexes was not used. Most important for the present study, the initial clearance of the cross-linked complexes was not faster than the clearance of non-cross-linked complexes (insert, Fig. 3).

*Glomerular and Extraglomerular Renal Localization of Immune Complexes.* For the initial experiment, NAP<sub>21,6</sub>·HSA-anti-NAP complexes were prepared at fivefold antigen excess and one half of the complexes were irradiated for cross-linking. No further purification steps were carried out. In SDS-PAGE analysis, 25.2% of the anti-NAP dissociated from the cross-linked complexes, comparable to previous findings on photoactivation of similar complexes (3). When these complexes were injected into mice and the left kidney removed at 4, 17, or 73 h by previously described procedures (8), the incompletely cross-linked complexes caused glomerular deposits that were less extensive than the deposits resulting from complexes that were non-cross-linked (Table I), as determined by immunofluorescence microscopy. Thus, these experiments suggested that when the majority of complexes were cross-linked, glomerular deposition was diminished from 4+ to 2+ both 4 and 17 h after injection. To prove that the deposits with both preparations were immunospecific, 50 mg of free NAP<sub>21,6</sub>·HSA

TABLE I  
*Glomerular Deposition of Non-cross-linked or Cross-linked NAP<sub>21,6</sub>·HSA-Anti-NAP Complexes that Were Not Further Purified, Detected by Immunofluorescence Microscopy\**

Time	Non-cross-linked complexes	Cross-linked complexes
<i>h</i>		
4	4+	2+
17	4+	2+
73	2+	±

\* Sections were stained with antibodies to rabbit IgG. By SDS-PAGE, 25.2% of the radiolabeled anti-NAP dissociated from complexes.

TABLE II  
*Glomerular and Peritubular Deposition of Non-cross-linked or Cross-linked, Further Purified NAP<sub>19</sub>·HSA-Anti-NAP Complexes, Detected by Immunofluorescence Microscopy\**

Time	Non-cross-linked complexes		Cross-linked complexes	
	Glomerular	Peritubular	Glomerular	Peritubular
<i>h</i>				
1	2+	++	2+	++
4	3.5+	++	1.5+	++
8	4+	+	±	+
24	4+	0	±	0
96	3.5+	0	±	0

\* Sections were stained with antibodies to rabbit IgG. By SDS-PAGE, 9.5% of the radiolabeled anti-NAP dissociated from complexes.

TABLE III  
*Detection of Glomerular Electron-dense Deposits after Administration of Non-cross-linked or Cross-linked NAP<sub>19</sub>·HSA-Anti-NAP Complexes\**

Time <i>h</i>	Non-cross-linked complexes		Cross-linked complexes	
	Subendothelial	Mesangial	Subendothelial	Mesangial
1	+	+	0	0
4	++	++	0	0
24	±	++	0	0

\* Presence of electron-dense deposits graded as ++ (extensive), + (few), ± (occasional), and 0 (none).

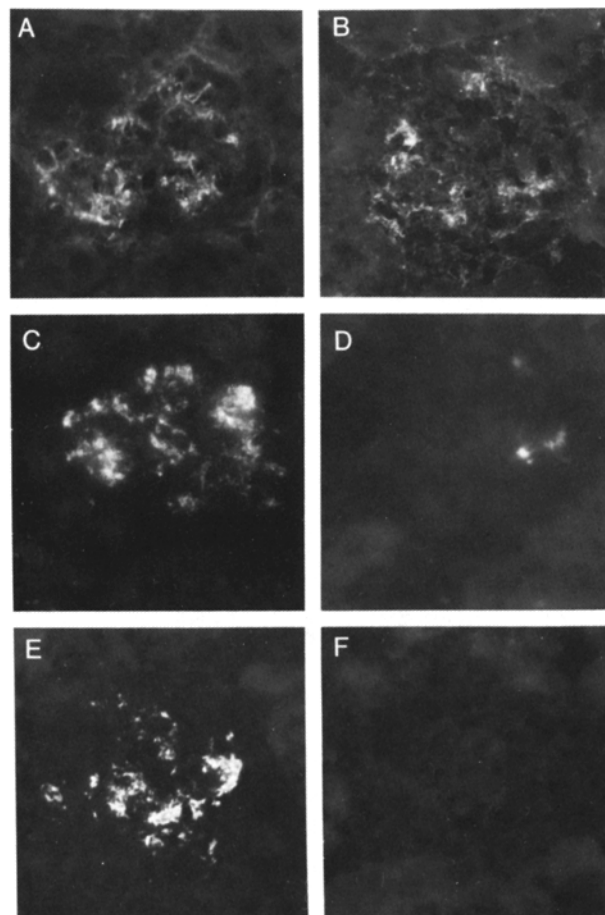


FIG. 4. Glomerular immunofluorescence patterns after staining with fluoresceinated antibodies to rabbit IgG in mice injected with non-cross-linked or cross-linked NAP<sub>19</sub>·HSA-<sup>125</sup>I-anti-NAP complexes. At 1 h the glomeruli of mice receiving non-cross-linked complexes (A) and cross-linked complexes (B) stained 2+. At 24 h the glomeruli of mice receiving non-cross-linked complexes (C) stained 4+ but the glomeruli of mice receiving cross-linked complexes (D) stained ± (trace). At 96 h the glomeruli of mice receiving non-cross-linked complexes (E) stained 3.5+ and were ± or negative in mice given cross-linked complexes (F).

was administered intraperitoneally after the completion of surgery for removal of the left kidney. The right kidney was removed 16 h later to determine if the excess antigen had solubilized the glomerular deposits as previously described (9). In all mice, the second kidney contained no deposits of rabbit IgG or antigen, as determined by immunofluorescence microscopy, indicating that the excess antigen had released the deposited complexes.

In the purified preparation of cross-linked NAP<sub>19</sub>·HSA-anti-NAP complexes, still 9.5% of the anti-NAP dissociated by SDS-PAGE analysis. The comparison of glomerular deposition of this preparation and the non-cross-linked control preparation revealed interesting differences (Table II). The non-cross-linked preparation showed 2+ glomerular deposits at 1 h, increased to 4+ deposits by 8 h, and declined by 96 h. The cross-linked complexes at 1 h also showed 2+ deposits, but these did not increase further, and from 8 to 96 h showed only trace ( $\pm$ ) deposits (Table III, Fig. 4). The staining for HSA in the NAP<sub>19</sub>·HSA paralleled the staining for rabbit IgG (data not shown). The glomeruli of mice given non-cross-linked complexes stained for mouse C3 as follows: 1+ at 8 h, 2.5+ at 24 h, 1+ at 96 h, and negative at earlier times. The glomeruli of mice given cross-linked complexes were negative for mouse C3 at all time points.

The injection of a single dose of the non-cross-linked NAP<sub>19</sub>·HSA-anti-NAP complexes also caused glomerular deposits that were 1.5+ at 1 h, 1+ at 8 h, and 1+ at 96 h, when stained for rabbit IgG. To rule out the glomerular deposition of NAP<sub>19</sub>·HSA alone, mice were injected repeatedly with 1.41 mg of the antigen alone, and by immunofluorescence microscopy glomerular binding of the antigen could not be found.

The sequence of observed events suggested that the non-cross-linked and cross-linked immune complexes initially became associated with glomerular structures in a comparable manner, as determined by immunofluorescence. The deposited non-cross-linked complexes persisted, but the cross-linked complexes failed to persist in glomeruli.

With non-cross-linked and cross-linked complexes, extensive fluorescence was seen adjacent to the peritubular capillaries in the renal cortex and medulla at 1 and 4 h after injection of the complexes, but by 8 h these deposits had diminished and by 24 h were totally absent (Fig. 5), as noted in an earlier publication (1). It was of interest that the fate of these deposits paralleled the fate of glomerular fluorescence after injection of cross-linked complexes (Table II).

Transmission electron microscopic studies were carried out on kidney specimens from mice at 1, 4, and 24 h after administration of non-cross-linked or cross-linked complexes. After administration of non-cross-linked complexes, only a few electron-dense deposits were seen at 1 h in subendothelial and mesangial areas. By 4 h subendothelial deposits had increased and extensive mesangial deposits were present (Fig. 6). By 24 h the subendothelial deposits had decreased and extensive mesangial deposits persisted (Table III). In contrast, after the administration of cross-linked complexes no electron-dense deposits could be identified in the 1, 4, or 24 h specimens (Fig. 6), even though immunofluorescence indicated that rabbit IgG and the NAP<sub>19</sub>·HSA molecules were present. These findings suggested that the cross-linked immune complexes had not rearranged or condensed into material visible as electron-dense deposits.

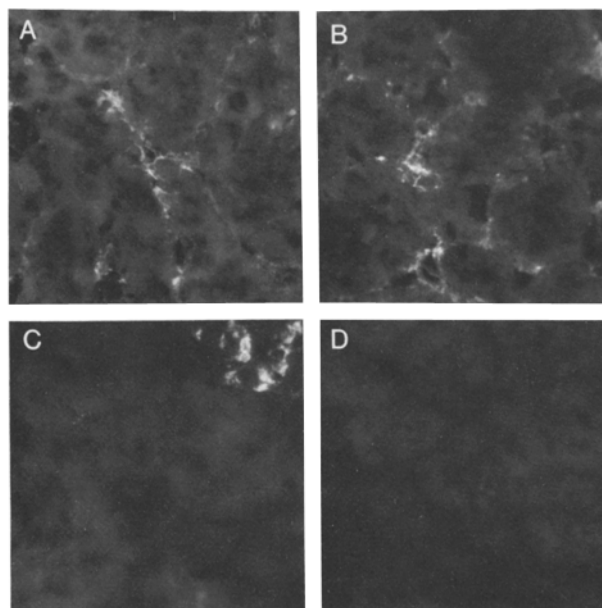


FIG. 5. Peritubular immunofluorescence patterns after staining with fluoresceinated antibodies to rabbit IgG in mice injected with non-cross-linked or cross-linked  $\text{NAP}_{10}$ -HSA-anti-NAP complexes. At 4 h the staining was ++ in mice that received non-cross-linked (A) or cross-linked (B) complexes. At 24 h no peritubular staining was found in capillaries after administration of non-cross-linked (C) or cross-linked (D) complexes. The positive staining in the right upper corner of (C) is glomerular staining that persists in mice given the non-cross-linked complexes.

The glomeruli of mice given non-cross-linked complexes had increased cellularity, as well as two to three polymorphonuclear leukocytes per glomerulus. The glomeruli of mice given cross-linked complexes also had increased cellularity but no increase in polymorphonuclear leukocytes.

The peritubular capillaries were carefully examined for electron-dense materials in the same specimens. Comparable electron-dense deposits were found at 4 h (Table III), both in the mice that received the non-cross-linked and cross-linked complexes (Fig. 7). These deposits were found outside of the tubular basement membrane and outside of the capillary lumen.

### Discussion

The present data indicate that circulating immune complexes, after initial attachment to glomerular structures, must undergo further rearrangement to persist in subendothelial and mesangial areas and to become detectable as electron-dense deposits. This conclusion was supported by the observation that the cross-linked and non-cross-linked immune complexes had comparable degrees of deposition at 1 h, as detected by immunofluorescence microscopy for the antibodies and antigen in the injected complexes. At this time point, sparse electron-dense deposits could be seen after injection of the non-cross-linked complexes, but were absent after injection of the cross-linked complexes. With passage of time the mice that received the non-cross-linked preparations showed increasing glomerular deposits by immunofluorescence microscopy and increasing electron-dense deposits, whereas in mice given the cross-linked complexes the deposits declined and electron-dense deposits did not evolve.



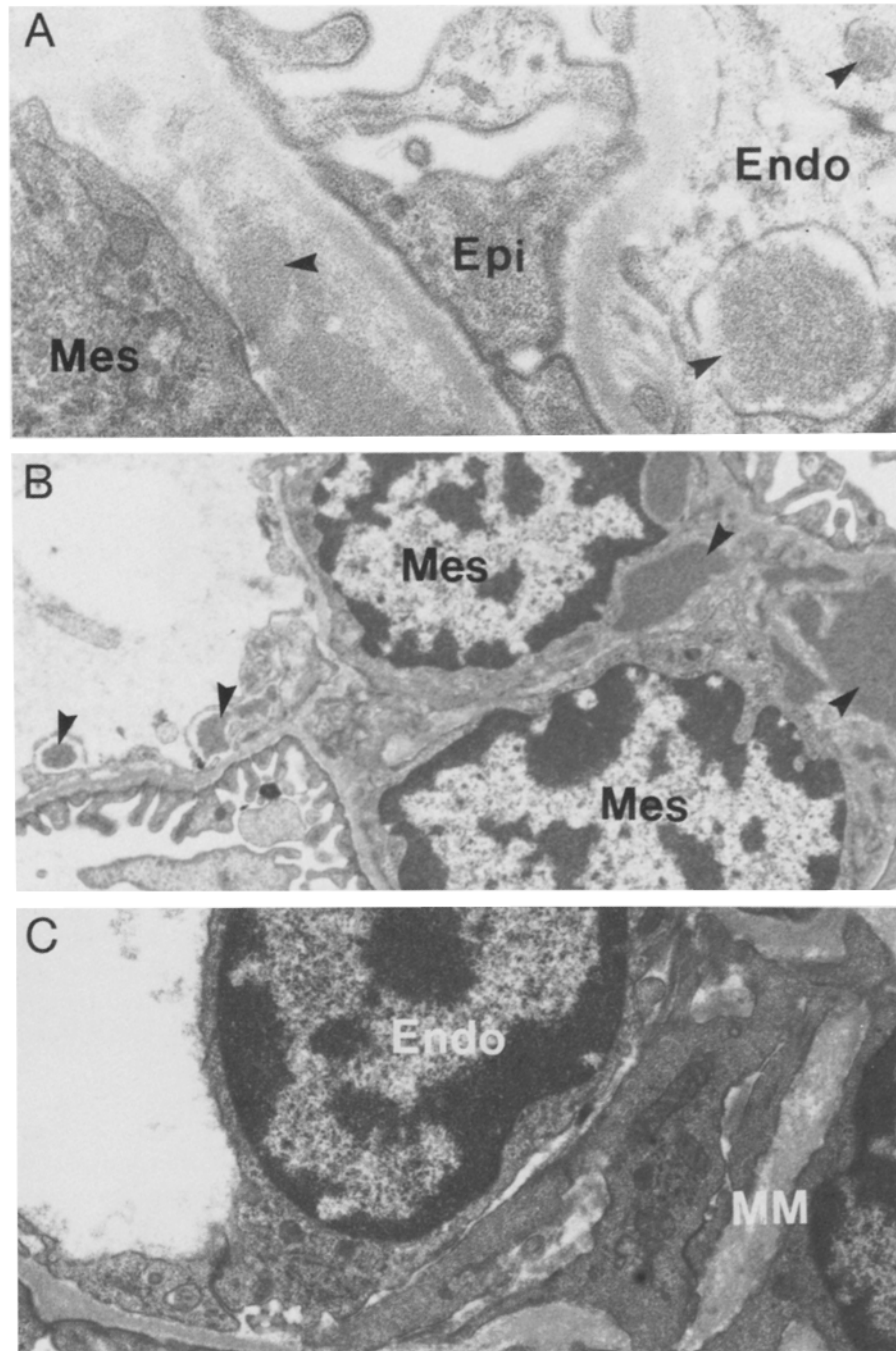


FIG. 6. Electron micrographs of glomeruli of mice injected with non-cross-linked or cross-linked NAP<sub>19</sub>-HSA-anti-NAP complexes. At 1 h in mice given non-cross-linked complexes (A) sparse electron-dense deposits (arrows) were present in association with endothelial cells and in mesangial matrix. At 4 h in mice given non-cross-linked complexes (B) subendothelial and extensive mesangial deposits were present. Mice given cross-linked complexes did not develop electron-dense deposits in glomeruli, as panel (C) shows for the 4 h time point. Mes, mesangial cells; Epi, epithelial cells; Endo, endothelial cells; MM, mesangial matrix. (A)  $\times 35,000$ ; (B)  $\times 12,000$ ; (C)  $\times 12,000$ .

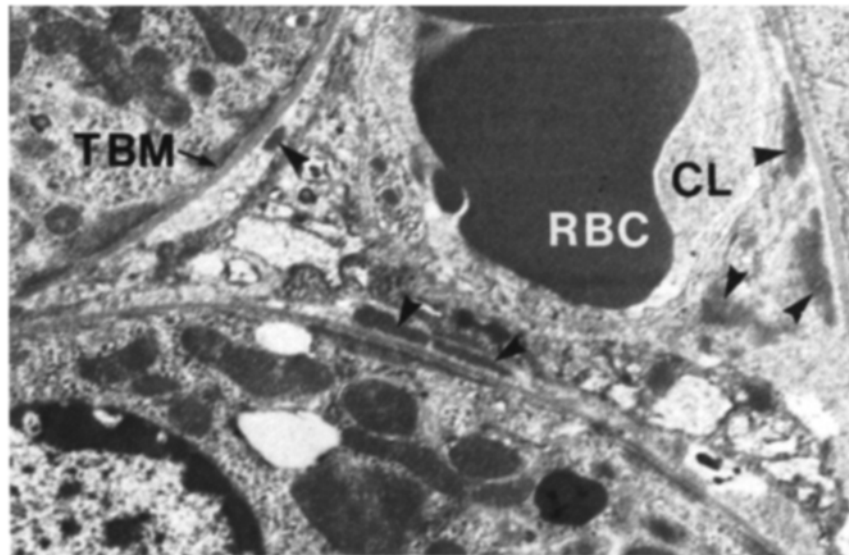


FIG. 7. Electron micrograph of a peritubular area of a mouse injected with non-cross-linked NAP<sub>19</sub>-HSA-anti-NAP complexes 4 h earlier. Electron-dense deposits (arrows) were present outside of the capillary lumen and adjacent to the tubular basement membrane. TBM, tubular basement membrane; RBC, erythrocyte; CL, capillary lumen.  $\times 10,000$ .

The trace amounts of deposits seen by immunofluorescence microscopy after the injection of cross-linked complexes were attributed to the residual antibodies (9.5%) that were not covalently cross-linked, since the reduction of free antibody from 25.2 to 9.5% by SDS-PAGE analysis reduced the persisting deposits from 2+ to trace ( $\pm$ ), as shown in Tables I and II. The inability of cross-linked immune complexes to rearrange *in vitro* was previously shown by the lack of precipitation and the absence of changes in lattice by gradient ultracentrifugation after the complexes had been purified and concentrated (3).

The mechanism for the initial attachment of cross-linked and non-cross-linked immune complexes to glomerular structures is not fully known. The electrostatic interaction between positively charged (cationic) antibodies and fixed negative charges in the glomerular basement membrane constitute one factor that promoted the initial interaction and subsequent development and persistence of electron-dense deposits in the subendothelial areas of the glomerular capillaries (10). Other factors very likely contribute to this initial interaction as well.

The further rearrangement of immune complexes in subendothelial and mesangial areas appears as a requirement for their persistence and condensation into electron-dense deposits. Antigen-antibody bonds that can rearrange are needed for this event since the cross-linked complexes did not persist or develop into electron-dense deposits. Furthermore, the development of the persisting deposits was immunospecific, since the deposits formed with partially cross-linked or non-cross-linked complexes were dispersed with administration of excess antigen as described in earlier studies (9). In addition, as noted by Kubeš (11), when mice were simultaneously immunized with ferritin and fibrinogen, each deposit contained only one antigen. The work of Michael *et al.* (reviewed in reference 12) showed that aggregated human IgG caused extensive

mesangial deposits in rats, suggesting that nonimmunospecific interactions can also lead to development of large mesangial deposits. It is noteworthy that extensive nonimmunospecific interactions were observed with aggregated human IgG (13). Interestingly, local rearrangement of large immune complexes was demonstrated as a requirement for phagocytosis by monocytes, using covalently cross-linked immune complexes (14). In the interaction with monocytes, binding of immune complexes to the Fc receptors constituted the process that caused increased local concentration of immune complexes that led to rearrangement to larger lattices and finally to interiorization. In glomeruli, locally increased concentration of immune complexes presumably occurred by electrostatic interactions for subendothelial deposit formation, as in a previously described model system (10). The potential role of glomerular filtration for the local increase of immune complexes for rearrangement has not been established.

The observed deposition of the non-cross-linked immune complexes was more extensive and the deposits persisted longer than in previously described results with complexes formed with HSA and antibodies to HSA at five times antigen excess (1). The increased deposition of complexes in the present data was in part due to the rapid removal of the excess NAP·HSA as previously described (4), leading to dissociation of circulating immune complexes and residual free antibody. The free antibody then could have combined with the free antigenic determinants in the complexes already deposited in glomeruli, as suggested for the chronic immune complex disease model induced with bovine serum albumin (15). The presence of free antibody, however, was unlikely to account for the proposed rearrangement and formation of electron-dense deposits since similar deposits occurred with the previously described HSA-anti-HSA complexes, made at five times antigen excess, where the free antigen persisted (1). Similarly, when complexes with cationized antibodies at five times antigen excess were administered, extensive deposits formed in spite of rapid removal of cationized antibodies, leaving free excess antigen in circulation (10). Therefore, we concluded that the free antibody contributed to the extensive deposits but was not solely responsible for the development and persistence of the electron-dense deposits in glomeruli.

It was of considerable interest that both the cross-linked and non-cross-linked immune complexes caused comparable deposits adjacent to peritubular capillaries by immunofluorescence. These deposits were transient and lasted a time period comparable to the glomerular deposits with cross-linked complexes (Table II). By electron microscopy these deposits were recognized at 4, but not at 1 h, both in the mice that received the cross-linked and non-cross-linked immune complexes. These electron-dense deposits were outside of the peritubular capillaries and outside but adjacent to the tubular basement membrane. The mechanisms of the formation of these deposits and the reasons for the transient existence of these deposits are not known at this time.

The observations that electron-dense glomerular deposits evolved after rearrangement of initially deposited immune complexes and that only the large deposits persisted indicate that the involved antigen-antibody system must have the potential to form precipitates. This conclusion suggests that the antigen-antibody systems involved in the formation of subendothelial and mesangial electron-dense deposits in human glomerulonephritides must also be caused by precipitating antigen-antibody systems among the circulating immune complexes. This concept should facilitate the

search and identification of circulating immune complexes that participate in the genesis of glomerulonephritides with subendothelial and mesangial deposits. In addition, the presented data indicate that some immune deposits detectable by immunofluorescence microscopy may not be identifiable as electron-dense deposits.

### Summary

Covalently, cross-linked immune complexes were prepared with multivalent 2-nitro-4-azidophenyl-human serum albumin (NAP-HSA) and antibodies to NAP at five times antigen excess. After purification with gel filtration, affinity chromatography with antigen-agarose column, and addition of the hapten, 9.5% of the antibodies dissociated from the complexes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. After injection of these cross-linked immune complexes into mice, glomeruli stained for the complexes by immunofluorescence microscopy for only a few hours and electron-dense deposits were not detected. In contrast, when the same immune complexes with comparable lattice but without covalent cross-linking were administered to a second group of mice, the initial deposition by immunofluorescence was comparable and then increased to extensive deposits that persisted to 96 h. In this second group of mice extensive electron-dense deposits evolved. These observations supported the conclusion that the immune complexes initially deposited from circulation must undergo rearrangement to persist and to form electron-dense deposits in glomeruli. The covalently cross-linked immune complexes existed in glomeruli only for a short period of time since these complexes could not rearrange.

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