

EFFECT OF CATIONIZED ANTIBODIES IN PREFORMED IMMUNE COMPLEXES ON DEPOSITION AND PERSISTENCE IN RENAL GLOMERULI*

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Renal glomeruli are an important site for deposition of immune complexes that cause inflammation and subsequent tissue damage. The glomerulus serves as a size and charge barrier for circulating macromolecules and presents a highly negatively charged (anionic) surface to the circulation (1). The role of the anionic charges on the binding, formation, and persistence of immune complexes in glomeruli has only recently begun to be examined. Gallo et al. (2) showed that immune complexes prepared with cationized antigen and cationized antigen alone bound to the anionic sites in the glomerular basement membrane within 1 h of administration, but later time points were not examined. Oite et al. (3) showed the binding of cationized antigen (Ab_{ED})¹ to the glomerulus and the formation of subepithelial deposits when antibody was administered subsequent to the binding of the antigen. Furthermore, antibodies to DNA eluted from glomeruli of NZB/W and MRL/1 mice were more cationic than those in circulation (4), suggesting that cationic antibodies may preferentially lead to deposition of immune complexes in glomeruli.

The proportion of naturally formed highly cationic antibodies is small and difficult to isolate in sufficient quantities to investigate their role in immune complex deposition. For these reasons, chemical cationization of antibodies was performed and antigen-binding ability was preserved. Immune complexes prepared with these cationized antibodies (AgAb_{ED}) demonstrated the important contribution of antibody charge to the glomerular deposition of immune complexes. The injected large-latticed AgAb_{ED} formed extensive subendothelial electron-dense deposits in mice.

Materials and Methods

Preparation of Antibodies and Antigen. Rabbit immunoglobulin G (IgG) was purified from rabbit fraction II (Miles Laboratories, Elkhart, IN) by gel filtration over Sephadex G-200 column (Pharmacia Fine Chemicals, Pharmacia Inc., Piscataway, NJ). Rabbit antibodies to human serum albumin (HSA) were isolated by affinity chromatography, and the monomeric fractions obtained by gel filtration were trace labeled with ¹²⁵I, all by previously described

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¹ Abbreviations used in this paper: Ab_{ED}, cationized antibody; AgAb_{ED}, immune complexes prepared with cationized antibody; HSA, human serum albumin; anti-HSA, rabbit antibody to human serum albumin; TCA, trichloroacetic acid; TNBS, trinitrobenzenesulfonic acid.

methods (5). HSA (E.R. Squibb, New Brunswick, NJ) was purified by DEAE ion exchange chromatography and gel filtration.

Sucrose Density Gradient Analysis. The monomeric status of the protein preparations and the size of immune complexes were characterized on 10–30% sucrose in borate-buffered saline gradients, run 37,000 rpm at 4°C for 16 h in a SW41Ti rotor and Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The samples were collected and analyzed for radioactivity on an automatic gamma counter (Searle Radiographics Inc., Des Plaines, IL).

Cationization of the Antibody. The reaction was carried out in a beaker in an ice bath. A typical experiment was as follows: 1.06 ml of ethylene diamine was added to 13.5 ml of 0.1 M sodium acetate buffer at pH 4.75, and the pH adjusted to 4.75 with concentrated HCl. The volume was brought to 20 ml with the same cold acetate buffer. The antibody preparation was added, 7.0 mg/ml in 18-ml vol of acetate buffer. The pH was readjusted as needed to 4.75. 605 mg of EDAC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide HCl (Sigma Chemical Co., St. Louis, MO) was allowed to dissolve and the reaction maintained at pH 4.75 for 30 min. The unreacted reagents were removed by exhaustive dialysis against 0.2 M sodium borate buffer, 0.15 M NaCl, pH 8.0.

The degree of substitution of ethylenediamine onto protein was assayed by spectrophotometric analysis for free amino groups using trinitrobenzenesulfonic acid (TNBS). The TNBS method as described by Habeeb (6) was modified by a 30-min incubation period at room temperature in 10% sodium dodecyl sulfate before the addition of 1.0 M HCl to facilitate the solubilization of cationized proteins, which tended to precipitate during the assay.

Isoelectric Focusing. Isoelectric focusing was carried out on a flatbed Pharmacia apparatus. A stock solution was prepared by mixing 2.42 g acrylamide with 0.75 g bis-acrylamide (electrophoretic grade; Bio-rad Laboratories, Richmond, CA) in 25 ml water and deionizing for 1 h with Amberlite ion exchange resin (Mallinckrodt Inc., St. Louis, MO) to remove the acrylic acid. To 15 ml of stock solution was added 1.9 ml ampholytes, pI range 3–10 (Pharmacia Fine Chemicals), 9.1 ml water, and 4 ml glycerol, and the mixture was thoroughly degassed under vacuum. The gel mold was flushed with nitrogen and 200 μ l of 22.8 mg/ml solution of ammonium persulfate, 30 μ l of TEMED (Biorad Laboratories) was added for polymerization and the gel was cast. Focusing was done at 20 W for 1.5 h at 15°C, and the gel was stained with Coomassie blue. The use of broad range (pI 3–10) standards (Pharmacia Fine Chemicals) allowed direct determination of the pI from the gel.

Preparation and Characterization of Immune Complexes. Complexes were prepared at fivefold antigen excess by weight based on equivalence points determined as the point where free antigen and free antibody were at a minimum in sucrose density gradient analysis. This was ~150 μ g of HSA/mg of antibody, which coincides with values obtained for unaltered anti-HSA determined by the precipitin reaction. Complexes were also prepared at 50-fold antigen excess. All complexes were prepared at room temperature and equilibrated for 4–5 d at 4°C before use. Their size distribution did not change during storage at 4°C over a 3-mo period.

Immunofluorescence. Tissues were prepared for immunofluorescence and evaluation as previously described (8). Staining was accomplished with one of the following fluoresceinated antisera: goat antibodies to rabbit IgG (Kallestad Laboratories, Austin, TX), goat antibodies to mouse IgG (N. L. Cappel Laboratories, Cochranville, PA), goat antibodies to HSA (Kallestad Laboratories and N. L. Cappel Laboratories), goat antibodies to mouse C3 (N. L. Cappel Laboratories). The specificity of the antisera was verified by use of purified rabbit IgG, mouse IgG, or HSA linked to agarose beads as previously described (9). The intensity of fluorescence was determined in a minimum of 10 glomeruli and graded 0–4+. Briefly, the standard grading system was as follows: 4+ glomeruli were readily visible at 40 \times magnification, and fluorescence was present throughout each glomerulus; 3+ were slightly visible at 40 \times , and fluorescence was present in all quadrants of glomeruli; 2+ glomeruli were visible at 100 \times and contained partial staining in 2–3 quadrants; 1+ glomeruli were dimly visible at 100 \times or contained isolated deposits; and trace positive glomeruli were visible only at 400 \times or contained deposits in <20% of the 10 or more glomeruli examined. In some experiments substantially heavier deposits were observed and differentiation between levels of 4+ staining was not possible with the defined criteria. Observations regarding tubular and interstitial fluorescence were also recorded. Pho-

tography was performed on a Zeiss epifluorescent microscope (Zeiss, Oberkochen, Federal Republic of Germany) with TriX Pan ASA 400 film (Eastman Kodak, Rochester, NY) with a 20-s exposure time.

Electron Microscopy. Samples of renal cortex were prepared for electron microscopy and processed as previously described (8).

Experimental Design. Female C57Bl/6J mice were used (The Jackson Laboratory, Bar Harbor, ME) and ranged in size from 18–22 g. Mice received KI in their drinking water at least 24 h before injection and throughout the experiment. All tail vein injections consisted of 0.5 ml of 0.2 M borate, 0.15 M NaCl, pH 8.0 buffer containing the designated protein.

For blood clearance kinetics, either 1 mg ^{125}I -Ab_{ED} or Ag ^{125}I -Ab_{ED} at fivefold antigen excess and containing 1 mg of ^{125}I -Ab_{ED} was injected. Retroorbital plexus blood samples of 10 μl were drawn at 1, 2, 4, 6, 10, 15, and 30 min, 1, 2, 4, 8, 12, 24, 48, 72, and 96 h. Samples were solubilized in 1 ml 0.1 M HCl and precipitated by the addition of 0.5 ml 22.5% trichloroacetic acid (TCA). After centrifugation, the pellet and supernatant were separated and assayed for radioactivity. Only TCA-precipitable material was considered to represent intact protein remaining in circulation. The percentage remaining in circulation was calculated from the amount injected and the blood volume of the mouse calculated from the relationship of 0.08 ml/g of body weight as previously determined (10). This method of analysis was necessary because of the removal of large fractions of the injected dose before the 1-min time point. Data for clearance of unaltered antibody and complexes prepared with unaltered antibodies have been previously described (11). Analysis of blood clearance curves was achieved by graphic subtraction of terminal components as previously described (12).

For glomerular localization studies, varying doses of unaltered antibodies, cationized antibodies, or immune complexes with one or the other antibody preparations were administered. Mice were killed at 1 min, 1, 12, 48, and 72 h, and 14 d after injection.

For glomerular localization of AgAb_{ED} made at 50-fold antigen excess, a 1-mg dose was administered, and mice were killed at 1 min, 1, 12, and 72 h after injection. To determine removal of complexes from glomeruli by excess antigen, four mice were injected with 1 mg AgAb_{ED} made at fivefold antigen excess. 12 h later, each received 10 mg of HSA intravenously. The mice were killed at 24, 36, 48, and 72 h after the injection of AgAb_{ED}.

Results

Characterization of Cationized Antibodies and Immune Complexes. The average number of modified amino groups in the anti-HSA ranged from 27 to 35. On isoelectric focusing these preparations exhibited a pI ≥ 9.3 . Only small amounts of unaltered or minimally altered IgG persisted (Fig. 1). Ab_{ED} readily precipitated with antigen and exhibited specificity for HSA. In the cationized anti-HSA, however, 21–25% of the antibody remained monomeric in antigen excess on sucrose density gradient analysis (Fig. 2), whereas only 10% of unaltered anti-HSA remained in this fraction. Thus, 11–15% of antibodies had been altered sufficiently to lose reactivity with antigen.

To determine if alterations other than the cationization could have contributed to the changes to be described, rabbit IgG was substituted with glycine by the carbodiimide procedure. In this manner, an average of 18.5 glycines were substituted per IgG molecule as determined by amino acid analysis (AAA Laboratories, Mercer Island, WA). With this substitution, the number of charged groups on IgG would not be altered, and in fact the pattern of IgG on isoelectrofocusing was not changed. The clearance from circulation of the radiolabeled, glycine-substituted IgG was not altered in comparison to native IgG, and no glomerular localization was found by immunofluorescence microscopy (data not shown).

Clearance Kinetics of Ab_{ED} and AgAb_{ED}. The blood clearance kinetics of Ab_{ED} were carried out to determine if the cationization substantially altered the fate of these molecules as compared with rabbit anti-HSA. The disappearance of a 1-mg dose of

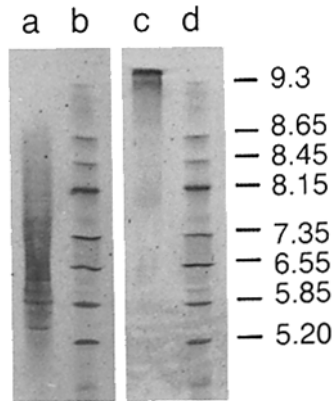


FIG. 1. Isoelectric focusing pattern of unaltered and cationized antibodies. In lane a, The unaltered anti-HSA shows the polydispersed pattern characteristic for polyclonal IgG. Lanes b and d show the standards. In lane c, The majority of Ab_{ED} had a $pI \geq 9.3$, with only a small amount of material exhibiting lower pI .

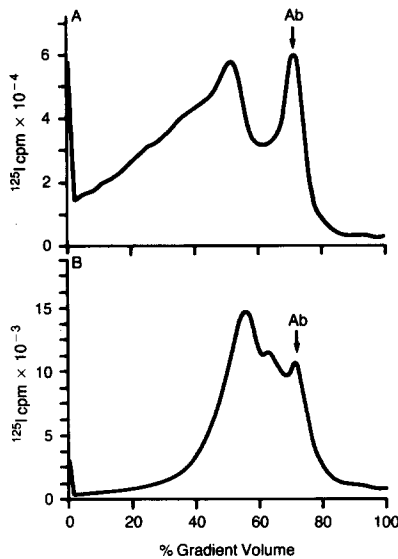


FIG. 2. Sucrose density gradient ultracentrifugation patterns of $Ag^{125}I-Ab_{ED}$. (A) Complexes were prepared at fivefold antigen excess and show 21% of the labeled antibody as unreacted monomer. (B) Immune complexes were prepared with the same Ab_{ED} at 50-fold antigen excess. The complexes prepared at 50-fold antigen excess are smaller than those prepared at fivefold antigen excess and are composed primarily of Ag_2Ab_2 and Ag_1Ab_1 , as previously described (7).

$^{125}I-Ab_{ED}$ was followed for a 96-h period. By 1 min after injection, only $53.9\% \pm 3.7\%$ of the injected material remained in circulation. The subsequent removal was best characterized by three exponential components. The terminal component accounted for 7.85% of the injected material and possessed a $t_{1/2}$ of 77.4 h, the same $t_{1/2}$ as the terminal component of unaltered anti-HSA (Fig. 3), and may represent only minimally cationized anti-HSA. The appearance of ^{125}I -labeled TCA-soluble material in circulation 30 min after injection of $^{125}I-Ab_{ED}$ indicated degradation of the rapidly removed Ab_{ED} . A similar burst of TCA-soluble radioactivity was not observed at any

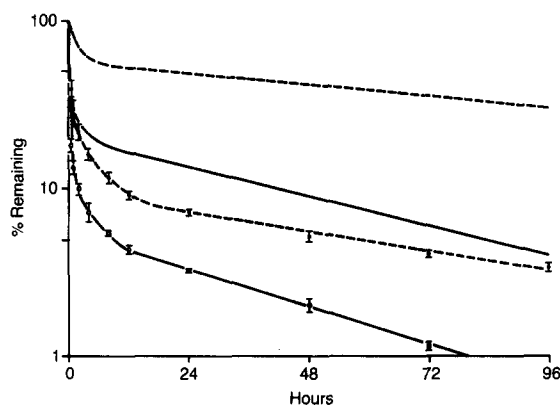


FIG. 3. Blood clearance kinetics of Ab_{ED} and $AgAb_{ED}$. The mean TCA-precipitable radioactivity on the antibodies was determined for three mice, and the curves were fitted by linear regression analysis. The clearance of Ab_{ED} alone (\bullet) is accelerated as compared with unaltered antibody (Ab) ($---$), but the final components are parallel suggesting a small percentage of unmodified or lightly modified Ab in Ab_{ED} . The clearance of $AgAb_{ED}$ at fivefold antigen excess (\square) is also substantially accelerated as compared with immune complexes prepared with unaltered Ab ($---$).

time point after the injection of unaltered antibody. Liver and kidney binding of ^{125}I -labeled material reached a maximum 1 h after injection and was 21.4% of the injected dose in the liver and 19.6% in the kidneys.

The blood clearance kinetics of $AgAb_{ED}$ were examined in a similar manner by injection of complexes prepared at fivefold antigen excess with 1 mg ^{125}I - Ab_{ED} contained in the dose. By 1 min after injection, only $38.8\% \pm 2.3\%$ of the material remained in circulation. The subsequent removal of radioactivity was best described by three exponential components. The terminal component accounted for removal of 4.35% of the injected dose and had a $t_{1/2}$ of 34.0 h (Fig. 3). This slow removal phase was comparable to that previously established for removal of similar immune complexes prepared with unaltered anti-HSA (11). The appearance of ^{125}I -labeled TCA-soluble material in circulation 30 min after injection of $Ag^{125}I$ - Ab_{ED} indicated the degradation of the rapidly removed ^{125}I - Ab_{ED} in the complexes. Liver and kidney binding of ^{125}I -labeled material reached a maximum 1 h after injection and was 28.3% of the injected dose in the liver and only 3.4% present in the kidneys. The slow removal of a portion of the injected complexes suggested that these were small-latticed complexes containing minimally cationized antibodies. To assure that the removal of immune complexes did not represent pooling of complexes in extravascular spaces, total body disappearance was determined. The data points at 72 and 96 h after injection of $AgAb_{ED}$ showed that the total body disappearance curve was parallel to the slow exponential component of clearance from the blood. At 96 h, 6% of the injected ^{125}I remained in the body and 0.6% remained in the circulation, thus providing no evidence for an unusually large reservoir of complexes containing cationized antibodies. The disappearance from circulation of $AgAb_{ED}$ with a 5-mg dose of antibodies was identical to that of the 1-mg dose.

Glomerular Localization Studies. In initial experiments, the renal localization of Ab_{ED} alone was examined by injection of 5 and 1 mg doses of Ab_{ED} , which were comparable to the largest doses administered as immune complexes (Table I). By 1 min, extensive deposition of Ab_{ED} was already noted (Fig. 4a). This high level of staining for rabbit

TABLE I

Time	Antibody dose							
	Ab	Ab _{ED}		AgAb	AgAb _{ED}			
	5.0	5.0	1.0	5.0	5.0	1.0	0.5	0.05
	<i>mg</i>							
1 min	0	4+	4+	1+	4+	4+	4+	4+
1 h	0	4+	4+	1+	4+	4+	4+	3+
12 h	0	2+	1+	2+	4+	4+	4+	2+
48 h	0	0	0	1+	4+	4+	3+	1+
72 h	0	0	0	1+	4+	4+	3+	1+
14 d	0	0	0	0-tr*	4+	4+	3+	tr

Glomerular staining for rabbit IgG after administration of either normal or cationized antibodies alone or as immune complexes was evaluated on a scale of 0-4+ staining.

* Trace positive glomeruli.

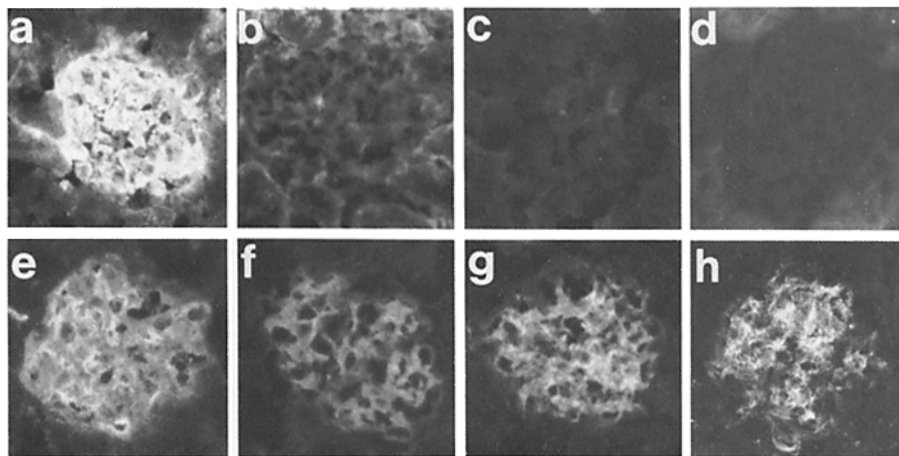


FIG. 4. Glomerular immunofluorescence patterns after staining with fluoresceinated antibody to rabbit IgG. The glomeruli of mice injected with 5 mg of Ab_{ED} alone stain intensely at 1 min (a) but only minimally by 12 h (b) after injection. At 72 h (c) and 14 d (d), the glomeruli are negative. When the AgAb_{ED} containing 5 mg of Ab_{ED} was administered, the glomeruli stain extensively at all time points. Staining is predominantly of peripheral loop patterns at 1 min (e) and 12 h (f), whereas at 72 h (g) and 14 d (h), mesangial staining is present as well.

IgG persisted through 1 h, decreased by 12 h (Fig. 4b), and had disappeared by 72 h after injection (Fig. 4c-d). The staining for rabbit IgG was extensive and primarily in a loop pattern. Controls with unaltered antibody showed no glomerular localization at any time point.

Administration of Ab_{ED} resulted in specific immunofluorescence in the tubules from 1 min to 72 h. At 1 min, Ab_{ED} could be detected in the lumen and proximal tubule brush border, whereas by 1 h, the staining of the cortex was extensive and obliterated all structural detail. This maximal staining of the renal cortex corresponded with the maximal uptake of ¹²⁵I-Ab_{ED} by the kidney as described above. By 12 h, the strongest staining persisted in the base of the tubular cells, and some degree of staining in this region persisted at 72 h but was absent by 14 d.

Significant glomerular staining for mouse C3 or mouse IgG was not detected until after 72 h. At later time points, deposition of these materials could be expected from an endogenous immune response to the injected protein.

Electron microscopy of animals receiving the 5-mg dose of Ab_{ED} revealed fine electron dense deposits at the anionic sites in the lamina rara interna and lamina rara externa of the basement membrane at the 1 min (Fig. 5 a) and 1 h time points (not shown). At 12 and 72 h (Figure 5 b, c), small spherical deposits were seen occasionally in the subendothelial regions. By 14 d the basement membrane area lacked any sign of deposits (Figure 5 d), but mesangial deposits were present despite the absence of positive immunofluorescence.

After the injection of the AgAb_{ED}, made at fivefold antigen excess, both rabbit IgG and HSA were detected by immunofluorescence microscopy in the glomeruli at 1 min (Fig. 4 e). Such deposits persisted through 14 days (Fig. 4 f-h). At dose levels of 5, 1, and 0.5 mg of antibody in AgAb_{ED}, this localization was extensive. When the dose was decreased to 0.05 mg, the initial deposits were 4+ and decreased at later time points (Table I). In comparison to the localization observed after Ab_{ED} alone, the immune complex deposits containing Ab_{ED} persisted longer (Table I). The patterns of immunofluorescence represented capillary loop deposition through 72 h with a granular pattern apparent in the 72-h animals. Mesangial deposition was prominent at 14 d with a residual loop pattern as well. Mouse C3 was not found by 72 h, but was present on the 14th d. Mouse IgG was found in large amounts on the 14th d, indicative of an endogenous immune response to the injected immune complexes.

Unlike the results obtained with free Ab_{ED}, tubular localization of antibody was not detected from 1 min to 12 h after the injection of AgAb_{ED}. After 12 h and through 14 d, small punctate deposits of antigen and antibody were seen in some tubules.

Electron microscopy of the glomeruli obtained 1 min after the 5-mg dose of AgAb_{ED} revealed some dense deposits at the anionic sites in the glomerular basement membrane (Fig. 5 e). This pattern persisted at the 1-h time point (not shown) with the appearance of a few formed deposits in the subendothelial areas. By 12 h, the anionic sites no longer contained deposits and larger dense subendothelial deposits were present (Fig. 5 f). At 72 h the subendothelial deposits were numerous and dense (Fig. 5 g). Small subepithelial deposits were present in some sections from 12 to 72 h. By 14 d the subendothelial deposits were absent and small, dense, well-defined subepithelial deposits were present (Fig. 5 h). Mesangial deposits were noted in samples from 12 h to 14 d with massive amounts present in the 14-d specimen.

Role of Lattice in Glomerular Deposition of AgAb_{ED}. Previous studies (13) have shown that small-latticed immune complexes prepared at large antigen excess do not deposit in glomeruli. To investigate the role of lattice on patterns of deposition and persistence of AgAb_{ED}, immune complexes were prepared at 50-fold antigen excess and confirmed to be of small lattice by sucrose density gradient analysis (Fig. 2 B). After injection of 1 mg of Ab_{ED} in immune complexes, initial deposits were noted in glomeruli as above, but by 12 h only sparse deposits remained (Fig. 6). Thus, the time course of removal from the glomeruli of AgAb_{ED} at 50-fold antigen excess was similar to the deposition and removal of Ab_{ED} alone. After the injection of the small-latticed AgAb_{ED}, HSA could also be detected in the glomeruli, indicating that the deposition was of immune complexes and not antibody alone.

The ability to release deposited AgAb_{ED} from glomeruli with excess antigen has

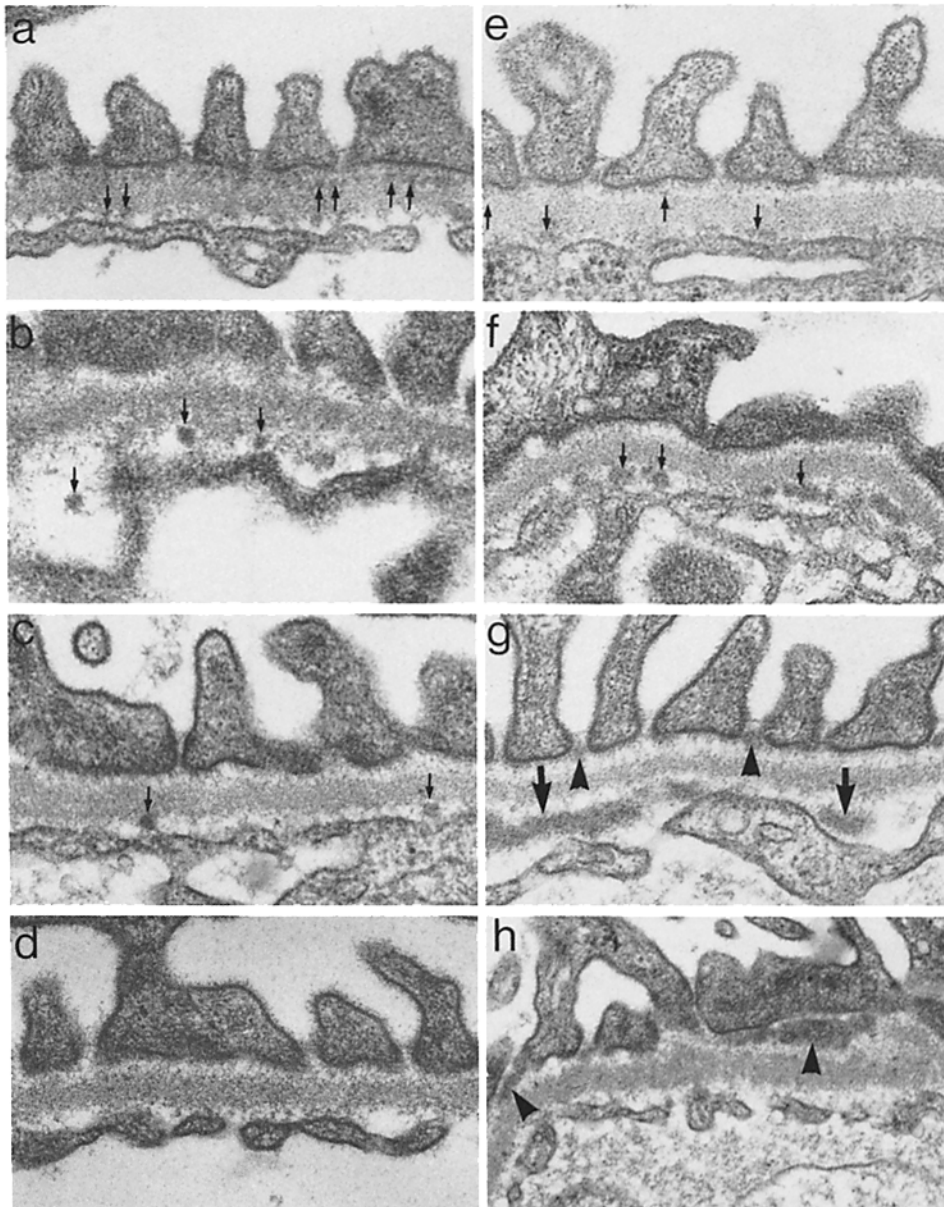


FIG. 5. Electron micrographs of glomerular capillaries. After a 5-mg dose of Ab_{ED}, electron-dense deposits are visible at the anionic sites in the lamina rara externa and lamina rara interna (arrows) at 1 min (a). Round deposits (arrow) are seen in the subendothelial region at 12 h (b). By 72 h (c), occasional round deposits (arrow) are still present, and by 14 d (d), the ultrastructure is normal. After administration of AgAb_{ED} containing 5 mg of Ab_{ED}, deposits are present at the anionic sites at 1 min (e), and in the subendothelial area at 12 h (f) and 72 h (g) (arrows). Occasional subepithelial deposits (arrowheads) are visible at 72 h (g). By 14 d (h), large subepithelial deposits (arrowheads) are noted.

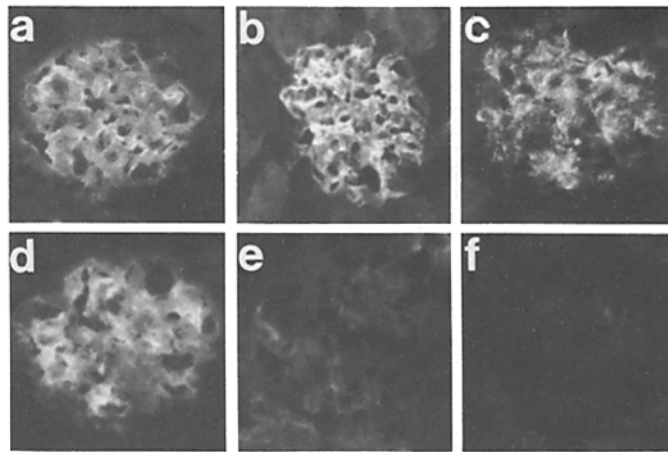


FIG. 6. Immunofluorescence patterns after staining with fluoresceinated anti-rabbit IgG. After injection of 1 mg of AgAb_{ED} prepared at fivefold antigen excess, the glomeruli stain intensely at 1 min (a), 12 h (b), and 72 h (c). After injection of a 1-mg dose of small-latticed AgAb_{ED} prepared at 50-fold antigen excess, the glomeruli stain intensely at 1 min (d), but by 12 h (e) and 72 h (f), the glomeruli are negative. Staining for the presence of antigen yielded similar patterns. These results indicate that small-latticed AgAb_{ED} localized but did not persist in the renal glomeruli.

been previously described (14). In similarly designed experiments, a 10-mg dose of HSA was administered 12 h after the injection of a 1-mg dose of AgAb_{ED} prepared at fivefold antigen excess. The intensity of immunofluorescence for rabbit IgG at 72 h was 2+ after the administration of excess HSA, compared with 4+ in control mice receiving the dose of AgAb_{ED} but not the HSA. Thus, the release of the deposited AgAb_{ED} from glomeruli was incomplete with the administration of excess antigen.

Discussion

With the methods used, extensive cationization of antibodies was achieved and the altered antibodies retained the capacity to form soluble immune complexes and immune precipitates. Up to 15% of the modified antibodies, however, lost the ability to combine with the antigen. Furthermore, the described alterations of the fate and glomerular localization of antibodies alone was attributable to the change in the isoelectric point and not due to other chemical alterations, as the substitution of a neutral amino acid by the same method causes neither increased disappearance from circulation nor glomerular localization of the modified antibodies.

The blood disappearance kinetics of Ab_{ED} and AgAb_{ED} were significantly accelerated over those seen with unaltered antibodies and immune complexes of comparable size. The very rapid initial clearance of Ab_{ED} before 1 min may be due to their adsorption to the negatively charged surfaces exposed to the circulation such as the endothelium, formed elements of the blood, and the glomerular basement membrane. The subsequent clearance could be attributable in part to glomerular filtration of the Ab_{ED} as evidenced by the appearance of material in the proximal tubule lumen at 1 min and the subsequent progressive penetration into the tubular cells. Hepatic uptake also contributed to removal of Ab_{ED} and AgAb_{ED}, but the mechanism for this was not elucidated.

Glomerular deposition of Ab_{ED} alone was extensive and extremely rapid, but these

deposits only persisted until ~12 h, a time point at which the Ab_{ED} were removed from circulation. These deposits were primarily at the anionic sites in the lamina rara interna and lamina rara externa of the glomerular basement membrane at the 1-min and 1-h times.

When Ab_{ED} were administered in the form of immune complexes, extensive glomerular deposition of antibodies and antigen, which persisted through 14 d, was shown by immunofluorescence. Even though both antigen and antibody were present in similar locations by immunofluorescent microscopy, the presence of some free Ab_{ED} alone in the observed electron-dense deposits cannot be excluded. By 12 h, large deposits were evident in the subendothelial area throughout the glomerular capillary loop. This finding was in contrast to the deposition of immune complexes with unaltered antibodies, which were found mainly in the subendothelial area adjacent to the mesangium and in the mesangial matrix (8). Furthermore, the extensive subendothelial deposits persisted at 72 h. Electron-dense deposits were also present in the mesangial matrix from 12 to 72 h. By immunofluorescence microscopy, extensive glomerular deposits developed even with a 0.05-mg dose of the AgAb_{ED}, indicating the propensity of the altered antibodies in immune complexes to localize in glomeruli.

The observations that both Ab_{ED} alone and AgAb_{ED} initially bound to the fixed negative charges in the glomerular basement membrane, but only immune complexes formed extensive subendothelial deposits along the loop of the capillary wall, suggests a sequence of events in the deposition of immune complexes in this location. Both of these materials bound only transiently to the anionic sites in the lamina rarae of the basement membrane and were released from these sites by as yet unknown mechanisms. The immune complexes, however, thereafter formed large deposits in subendothelial areas, presumably due to their ability to rearrange to form large lattices that then remained in the subendothelial location. The described experiments did not distinguish whether the complexes that evolved in the subendothelial areas were formed from the initially deposited material from the lamina rara externa, lamina rara interna, or both. It is unlikely that the subendothelial deposits developed from complexes still in circulation, since the bulk of the complexes (90%) was no longer present in circulation at 1 h, when only few subendothelial deposits were noted. Thus, the sequence of observed deposits supports the hypothesis that indeed the extensive subendothelial deposits evolved from the complexes that had initially bound to the fixed negative charges in or at the basement membrane. The subsequent fate of the subendothelial immune complexes was not delineated.

The recorded observations 14 d after the injection of AgAb_{ED} showed that both the antigen and antibody were still present. In addition, large quantities of mouse IgG and mouse C3 were also present, indicative of the endogenous immune response to the injected antigen, antibody, or both. At this long interval, the electron-dense deposits were both in subepithelial and mesangial areas, and not in the subendothelial area. The subepithelial deposits at this time may well have developed by *in situ* formation of immune complexes, consisting of mouse antibodies and the injected HSA, rabbit anti-HSA, or both as antigens. It is of interest that the prior deposition of cationized antigen in the glomerular basement membrane, followed by antibody injection, resulted in subepithelial deposits with no mesangial or subendothelial deposits (3). The mesangial deposits noted at 14 d may have arisen from the subendothelial deposits after the endogenous immune response, but direct mesangial

deposition of residual circulating HSA and rabbit anti-HSA by newly synthesized mouse antibodies cannot be ruled out. The presence of extensive rabbit IgG, however, supports the possibility that subendothelial deposits evolved into mesangial deposits, because at 96 h only $0.6 \pm 0.1\%$ of the radiolabel was present in circulation, and would have contributed little to mesangial deposition by endogenous immune clearance.

The absence of mouse C3 deposits in glomeruli up to 72 h after injection of AgAb_{ED} suggested that these antibodies were inefficient in the activation of complement. This was confirmed *in vitro* by the failure of these complexes to activate human complement (unpublished observation).

Previous studies showed that only large-latticed, preformed, circulating immune complexes deposited in renal glomeruli (8, 13). AgAb_{ED} prepared at 50-fold antigen excess, consisting of small-latticed (Ag₂Ab₂ and Ag₁Ab₁) complexes, deposited and persisted in glomeruli comparable to the Ab_{ED} alone. This observation could be explained by the hypothesis that immune complexes capable of cross-linking into larger deposits were necessary for the persistence in glomeruli. The already deposited complexes in the glomeruli were incompletely removed by the administration of excess antigen, in contrast to the complete release of complexes prepared with unaltered antibodies (14). This finding indicated that the charge of antibodies in complexes contributed to their retention and persistence in the glomeruli.

This study demonstrated the role of antibody charge in glomerular localization of immune complexes. The net charge of an immune complex as a whole is undoubtedly the most important parameter in this localization. Immune complex net charge will depend on the charge of the antibody and antigen and the nature of their union, rather than the simple arithmetic net charge of the complex. In the results herein, the antibodies were cationized and then combined with HSA, an anionic protein. The resulting complexes bound to anionic sites in glomeruli, forming extensive subendothelial deposits that persisted. In the work of Ebling and Hahn (4), cationic anti-DNA antibodies were eluted from NZB/W and MRL/1 kidneys, thus supporting the hypothesis that charge plays an important role in the *in vivo* localization, formation, and persistence of immune complex deposits in glomeruli.

Summary

To study the interaction of positively charged antibodies in immune complexes with the fixed negative charge on the glomerular capillary wall, chemical cationization of antibody was accomplished with the maintenance of antigen-binding activity. These cationized antibodies bound rapidly to glomeruli but did not persist. Large-latticed immune complexes formed with these cationic antibodies showed rapid deposition and persistence in renal glomeruli, even when administered in small doses. Electron-dense deposits were present at the anionic sites in the glomerular basement membrane at 1 min and 1 h, with extensive subendothelial deposits present from 12 to 72 h. By 14 d, the deposits were seen in the subepithelial region and the glomerular mesangium. The administration of small-latticed immune complexes prepared with cationized antibody revealed initial deposition without persistence in glomeruli in a manner similar to cationized antibodies alone. Thus, the positive charges on antibodies in immune complexes contribute to the deposition and persistence of the complexes

in glomeruli, particularly in the subendothelial area.

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