NONDISSOCIATING CATIONIC IMMUNE COMPLEXES CAN DEPOSIT IN GLOMERULAR BASEMENT MEMBRANE

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Considerable debate has centered recently on the mechanism(s) by which immune complexes (IC)\textsuperscript{1} lodge in glomerular basement membrane (GBM). The traditional view, namely that soluble IC deposit as such from the circulation, stemmed from observations that the initiation of glomerulonephritis in experimental serum sickness coincides with the period of immune elimination of antigen, the occurrence of IC in the circulation, and the presence of both antigen and antibody in glomeruli (1, 2). However, the relevance of these observations for clinical glomerulonephritis has been challenged for several reasons: there is often a poor correlation between the presence of IC in the circulation and in glomeruli, most notably in patients with membranous nephropathy characterized by diffuse subepithelial deposits; soluble IC prepared in vitro and passively administered to experimental animals tend to lodge in the renal mesangium (Mes), and with but few exceptions (3, 4) fail to deposit in the GBM; and IC formation is a dynamic process in which a state of equilibrium between dissociated and undissociated IC exists, allowing for the possibility that dissociated antigen or antibody could act independently of intact IC (5). An alternative view (6), namely that IC in GBM result from in situ binding of antibody to structural or previously "planted" antigen, is based on evidence that GBM deposits similar to those in human membranous nephropathy can be induced by passive transfer of serum from rats with autologous IC glomerular disease (Heymann nephritis) induced by immunization with tubular epithelial cell brush border antigen (7–9) and that similar deposits can result from perfusing the kidney with antibody to proximal tubular epithelial cell brush border antigen under conditions that essentially exclude circulating IC (10). Also, in situ complexing of antibody to planted antigens bound in the GBM on the basis of electrical charge has been demonstrated (11). Although the mechanisms by which IC localize in glomeruli have often been discussed in "either/or" terms, it should be emphasized that the two mechanisms are not mutually exclusive.

In a previous study soluble IC, prepared in vitro from cationic antigen and antibody and passively injected into mice, gave rise to GBM deposits (3). The IC

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\textsuperscript{1} Abbreviations used in this paper: BGG, bovine gamma globulin; BSA, bovine serum albumin; DEAE, diethylaminoethyl; GBM, glomerular basement membrane; IC, immune complex(es); Mes, mesangium; PBS, phosphate-buffered saline; RGG, rabbit gamma globulin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TDI, toluene-2,4-diisocyanate.
in subendothelial and subepithelial sites correlated with the known distribution of anionic sites described by others (12, 13) and suggested that cationic IC could be trapped electrostatically on a nonimmunological basis. Mechanistically, the evidence was consistent with deposition of intact IC from the circulation. However, because the cationic antigen by itself could bind to the GBM, the IC had been prepared in antigen excess to achieve solubility, and antigen-antibody complexes are to some extent dissociable, an in situ mechanism in which free antigen was first deposited with subsequent binding of free antibody, or IC with free antibody-combining sites, could not be entirely excluded (3). To examine directly whether intact, circulating IC per se can cross and deposit in the GBM, covalently linked, hence nondissociating, antigen-antibody complexes were used in the present study.

Materials and Methods

Animals. Male BALB/c AnNCrI BR mice, 6–8-wk old, were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA.

Antigens and Antisera. Bovine gamma globulin (BGG; Sigma Chemical Co., St. Louis, MO) was further purified by passage through Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ) in phosphate-buffered saline (PBS). The IgG fraction of rabbit anti-BGG antiserum (3) was prepared by precipitation in half-saturated (NH₄)₂SO₄ followed by passage through a diethylaminoethyl (DEAE)-cellulose (Schleicher & Schuell, Inc., Keene, NH) column in 0.0175 M potassium phosphate, pH 6.3, and gel filtration on Sephadex G-200 in PBS. Rhodamine and fluorescein-conjugated IgG fractions of goat anti-rabbit IgG, fluorescein-conjugated IgG fraction of rabbit anti-bovine IgG, fluoresceinated goat anti-mouse C3 and fluoresceinated goat anti-mouse IgG were obtained from N.L. Cappel Laboratories, Cochranville, PA. Affinity-purified and biotinylated goat anti-rabbit IgG and horseradish peroxidase-avidin-D were obtained from Vector Laboratories, Inc., Burlingame, CA.

Covalent Immune Complexes. BGG and rabbit anti-BGG were covalently linked in a two-stage reaction employing a bifunctional reagent, toluene-2,4-diisocyanate (TDI) (Eastman Kodak Co., Rochester, NY), whose groups are activated at different pH (14). BGG (1.5%) in 0.05 M potassium phosphate, pH 7.5, was reacted with 2% (vol/vol) TDI for 30 min at room temperature, cooled to 0°C for 15 min, centrifuged at 3,000 g for 15 min to remove solid TDI, passed through a Sephadex G-25 column in 5 mM phosphate, pH 7.5, to remove any residual TDI, and left at 0°C for 1 h. To the TDI-activated BGG an equal volume and concentration of the IgG fraction of rabbit antiserum to BGG, in the same buffer, was added dropwise with stirring and left for 1 h at 37°C to allow the formation of IC. By quantitative immune precipitation (15) it was determined that the IC were prepared in 70-fold antigen excess. To activate the second functional group to induce covalent cross-linking between antigen and antibody, the pH was raised to 8.5 by adding solid tribasic sodium phosphate. After 10 min the pH was lowered to 7.5 with solid monobasic sodium phosphate, and the solution was concentrated by ultrafiltration. The IC were then applied to a Sepharose 6B column in PBS containing 1 M NaCl to separate IC from noncomplexed IgG. The relevant peak was concentrated and then repurified on the same Sepharose 6B column. As controls, identical procedures were carried out with mixtures of BGG and BGG; anti-BGG and anti-BGG; BGG and normal rabbit gamma globulin (RGG).

For some experiments covalently linked IC were cationized or anionized (3). In other experiments BGG was cationized before covalent linkage with unmodified, native anti-BGG.

Analytical Procedures. SDS-PAGE was performed in vertical slabs, 3% stacking gel, 5% running gel, 0.1% SDS (Bio-Rad Laboratories, Richmond, CA) (16). Agarose (1%) gel electrophoresis was done at pH 8.6 in 0.06 M sodium barbital buffer. Sedimentation
velocity was performed in a Spinco Model E ultracentrifuge (Beckman Instruments, Inc.,
Spinco Div., Palo Alto, CA) with ultraviolet optics at a protein concentration of 0.8 mg/
ml in PBS.

Injection Protocol. Four groups of mice (8–10 mice per group) were sacrificed 1 h after
tail vein injection with 1 mg of covalently linked IC in 0.4 ml of saline: Group 1, cationic
IC, chemically modified after covalent coupling; Group 2, cationic IC from chemically
modified cationic BGG covalently linked to unmodified native rabbit anti-BGG; Group 3,
native IC, both BGG and rabbit anti-BGG native, unmodified; Group 4, anionic IC,
chemically modified after covalent coupling.

Morphology. Portions of kidney were processed and examined for immunofluorescence
and electron microscopy as previously described (3). For immunoelectron microscopy,
modifications of previous methods (17, 18) were employed. Kidney slices were fixed in
5% formalin in 0.1 M phosphate, pH 7.4, for 6 h. After washing in buffer for 3 h, then
overnight in cold 0.1 M Tris-Cl, pH 7.4, containing 1% bovine serum albumin (Tris-
BSA), the tissue was soaked for 30 min in 0.1 M Tris-Cl containing 10% dimethyl
sulfoxide, and snap frozen. Cryostat sections were washed in Tris-BSA, PBS-BSA, and
suspended in PBS-BSA containing 15 μg/ml biotinylated anti-rabbit IgG for 2 h. After
washing in PBS-BSA and 0.1 M NaHCO₃-BSA, sections were incubated in NaHCO₃-BSA
containing 50 μg/ml peroxidase-avidin for 2 h. After rinsing and development of the
peroxidase reaction, sections were flat embedded in Epon (17, 18). Glomeruli with cut-
open loops (to maximize access of reagents) were studied.

Results

Covalent Immune Complexes. As shown previously (14), the method of covalent
coupling promotes linkage of antigen to antibody, but not self-linkage of antigen
to antigen or antibody to antibody. This was found to be true in the BGG-anti-
BGG system as well since the method yielded covalently linked aggregates only
when antigen and antibody were present together. The gel filtration pattern
after the formation of covalent IC is shown in Fig. 1. Complexes demonstrated
to be separated from nonspecific aggregates and monomeric IgG were used for
subsequent experiments. On sodium dodecyl sulfate–polyacrylamide gel electro-

![Figure 1](image-url)

**Figure 1.** Gel filtration through a 50-cm × 1.5-cm column of Sepharose 6B in PBS (1 M
NaCl) after formation of covalent IC. Fractions of 1.0 ml were collected. Molecular weight
standards were blue dextran (BD), a human IgM myeloma protein, and BGG. The fraction
indicated by the double-headed arrow, tubes 20–32, was repassed through the same column
and used for subsequent experiments.
phoresis (SDS-PAGE) (Fig. 2), the purified IC in lane 3 were too large to enter the running gel, and were not contaminated with unbound or dissociable monomeric IgG or small aggregates. In contrast, the protein in control mixtures such as BGG and normal RGG (lane 2), which had been treated in the same manner as the IC, did not form large aggregates and therefore wholly entered the running gel. The fact that the IC did not dissociate in the presence of detergent confirms their covalent nature.

As demonstrated by a continuous precipitation line after double immunodiffusion in gel, the covalent complexes contained both BGG and rabbit anti-BGG linked together (Fig. 3).

The covalent IC that formed between cationic antigen and native antibody and were purified by gel filtration were analyzed by sedimentation velocity. The complexes were broadly heterogeneous, as would be expected, with an average $s_{20,w} = 28S$. 

**Figure 2.** SDS-PAGE of purified covalent, unreduced IC (lane 3), that fail to enter the running gel. Lane 1 contains standards: human secretory IgA, BGG, and F(ab')2 fragment of rabbit IgG. Lane 2 has a control mixture of BGG and normal RGG (instead of rabbit antibody to BGG) treated in the same way as the IC; the IgG is predominantly monomeric, and all protein enters the running gel. Other controls of BGG plus BGG or rabbit anti-BGG plus rabbit anti-BGG gave the same pattern as lane 2.

**Figure 3.** Characterization of covalent IC by double immunodiffusion in agarose gel. The center well on the left contains IC and the top wells contain goat anti-BGG and goat anti-RGG. The center well on the right contains a control mixture of BGG and normal RGG treated in the same manner as the IC. The continuous precipitation line in the left pattern indicates that the BGG and RGG are linked together; compare with the double spur in the control (right pattern).
Appropriate chemical modification of the covalently linked IC produced distinctly cationic or anionic complexes as shown by their migration in agarose gel electrophoresis (Fig. 4, top and bottom). Cationization of the BGG before covalent linkage with native unmodified rabbit anti-BGG also resulted in cationic IC, well 2, but with a lower net charge than complexes in which both antigen and antibody were cationized.

Renal Morphology. After intravenous injection, the two types of cationic IC (those with antigen and antibody both modified, or those with only antigen modified) deposited diffusely and uniformly in the GBM of the peripheral capillary walls, since both BGG and RGG were detected in the same distribution by immunofluorescence (Fig. 5 and Table I). There was some variability in the intensity of staining among animals, but no consistent difference between the...
FIGURE 5. Immunofluorescence for BGG (left) and RGG (right). Glomeruli from mice receiving covalent IC containing cationic BGG and cationic anti-BGG, group 1 (A and B), or cationic BGG and native anti-BGG, group 2 (C and D), demonstrate codeposition of antigen and antibody in peripheral capillary loops. In contrast, glomeruli from mice given covalent IC prepared from native BGG and native anti-BGG, group 3 (E and F), or anionic BGG and anionic anti-BGG, group 4 (G and H), show deposits in Mem but none in peripheral loops. × 300.
Table I
Glomerular Localization of Intravenously Injected Covalent Immune Complexes

| IC Group* | Immune reactants‡ | Ultrastructural deposits‡ |
|-----------|-------------------|--------------------------|
|           | No. of mice | BGG | RGG | No. of mice | Subepithelial | Subendothelial | Mesangial |
| 1—Cationic | 10         | 1-3+ GBM | 1-3+ GBM | 9 | 1-2+ | 1-3+ | 1+ |
| 2—Cationic BGG/Native | 10         | 1-3+ GBM | 1-3+ GBM | 5 | 1-2+ | 1-3+ | 1+ |
| Anti-BGG   | 3—Native    | 8    | 1+ Mes | 1+ Mes | 2 | 0    | 0    | 2+ |
| 4—Anionic  | 10         | 0-1+ Mes | 0-1+ Mes | 7 | 0    | 0    | 0-2+ |

* In groups 1 and 4, cationization and anionization were done after covalent complex formation. In group 2, the BGG antigen was cationized before IC formation; the rabbit antibody to BGG was unmodified. In group 3, both antigen and antibody were unmodified.

‡ The variability of intensity of immunofluorescent staining and number and size of ultrastructural deposits are given as a range on a scale of 0 to 3+. In group 4, half the mice showed no detectable antigen or antibody.

two cationic groups. Ultrastructurally, in both cationic groups there were discrete deposits diffusely in the subepithelial and subendothelial layers of the GBM (Fig. 6 and Table I). There was variation in size and number of deposits in loops within the same glomerulus; in some loops the deposits were of similar size and number in both layers while in other loops the deposits were larger and more numerous in the subendothelial than in the subepithelial layer. The latter difference was observed more frequently in group 2 than in group 1. Occasionally deposits were seen in the lamina densa. Small deposits were present in the mesangial matrix along the luminal front and at the junction of the mesangium and capillary wall in both cationic groups.

In mice given unmodified or anionic covalent IC (groups 3 and 4), the findings differed from those given cationic IC in that none showed GBM deposits. By immunofluorescence microscopy both BGG and RGG, restricted to the mesangium, were found in all of the mice in group 3 (native IC) and in half the mice in group 4 (anionic IC). Ultrastructurally, electron-dense deposits were present within the mesangial matrix but not in the GBM.

The immunoelectron microscopic studies confirmed the other observations in that both cationic groups, but not the native, anionic or control groups, had the enzyme reaction product marking the site of RGG antibody in the GBM (Fig. 7). It should be especially noted that even group 2 complexes containing native antibody were able to penetrate to the subepithelial layer. RGG was detected not only in subepithelial and subendothelial layers, but also in the lamina densa, which by ordinary electron microscopy has a greater density than the laminae rarae, thus obscuring less dense small IC.

None of the mice in any group showed staining for mouse C3; mouse IgG, present in the mesangium in background amounts, was similar in all groups.

Discussion

Our previous study showed that injected preformed soluble cationic, but not anionic, IC can give rise to GBM deposits in subendothelial and subepithelial
FIGURE 6. Electron micrographs of peripheral GBM of mice given covalent IC. In mice given cationic IC, group 1 (A) and group 2 (B), there are electron-dense deposits in subepithelial and subendothelial sites (examples indicated by arrows). No deposits are seen in mice given native, group 3 (C), or anionic, group 4 (D), IC. In all the micrographs the epithelium and endothelium are at the top and bottom respectively. Double stained with uranyl acetate and lead citrate. × 78,000.
FIGURE 7. Immunoelectron micrographs of peripheral GBM of glomeruli incubated with biotinylated anti-RGG and peroxidase-avidin. In mice given covalent IC containing cationic BGG and native anti-BGG, group 2, RGG antibody labeled by the black enzymatic reaction product is present in all layers of the GBM (A). Reaction product is absent from the GBM of mice given covalent IC containing native BGG and native anti-BGG, group 3 (B), and from controls given cationic BGG and normal RGG (C). Unstained sections. × 78,000.
sites and emphasized the potential importance of charge as a determinant of IC localization (3). Exploiting this tendency of cationic IC to deposit in GBM, in the present work we used soluble, but nondissociating complexes to determine whether IC in the circulation can cross the GBM as intact complexes and deposit in subepithelial sites, a point that has been difficult to establish in passive serum sickness and which has led in part to doubts of the importance of circulating IC in common forms of clinical glomerulonephritis. The complexes with the ability to localize along both aspects of the GBM contained BGG antigen and rabbit anti-BGG antibody covalently linked together, as indicated by SDS-PAGE and double immunodiffusion, were large (average sedimentation coefficient of 28S), and had a net positive charge as shown in agarose gel electrophoresis, which minimizes electroendosmosis. Accordingly, conditions required for in situ IC formation in the GBM, as initiated by planted or intrinsic antigen, were essentially eliminated. The observation that relatively large, covalent, cationic IC were able to lodge in the GBM strongly suggests that intact IC, under appropriate conditions, have the potential to cross the GBM and deposit in subepithelial sites, and are not necessarily excluded by size alone. Such a mechanism is fully consistent with the traditional concept of glomerular IC formation in active serum sickness, where glomerular lesions were found predominantly in animals forming large IC, greater than 19S in size (19, 20).

Evidence that parallel exclusion of IC can occur on the basis of charge derives from the observation that covalently linked IC with a negative charge did not deposit in the GBM. This result supports our previous conclusions concerning the potential importance of IC charge in the formation of GBM deposits (3).

While the evidence presented clearly demonstrates that intact, sufficiently cationic IC can directly deposit in the GBM from the circulation, it should be pointed out that the significance of the current and related findings for human glomerulonephritis remains to be established. Nevertheless, it is well known that the immune response is usually heterogeneous with respect to the charge of the antibodies formed, and natural antigens can likewise vary in charge. Thus, conceptually it is plausible that in a situation where IC of widely diverse net charges are circulating, those complexes on the very cationic end of the spectrum, which may be but a small fraction of the total, could be the ones that lodge in the glomerulus and cause disease. In the present study the group 2 IC composed of cationized antigen and native antibody could be especially relevant.

Summary

The mechanisms by which immune complexes deposit in the glomerular basement membrane have been the subject of much debate, with the relative importance of direct deposition of circulating immune complexes (IC) vs. formation of IC in situ from the binding of circulating antibody to structural or exogenous planted antigen being at issue. In order to determine whether intact IC can deposit as such, covalently linked IC were prepared by a two-step reaction involving the bifunctional reagent toluene-2,4-diisocyanate (TDI), the antigen bovine gamma globulin (BGG), and rabbit anti-BGG antibody. Antigen and antibody were covalently cross-linked, with little self-linkage of antigen or antibody, and IC were purified by gel filtration. The net charge of the complexes
was varied by chemical means, either before or after IC formation. When cationic IC were injected intravenously into mice, there was codeposition of antigen and antibody diffusely in the glomerular basement membrane (GBM), and deposits were observed ultrastructurally in the laminae rarae, interna and externa, and the lamina densa. Thus, under conditions of restricted appropriate charge, intact IC can cross the glomerular basement membrane and deposit in subepithelial sites without being excluded by size alone.

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