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Interaction of immune complexes with glomerular heparan sulfate–proteoglycans

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Interaction of immune complexes with glomerular heparan sulfate–proteoglycans. The binding characteristics of cationic and more neutral immune complexes with heparan sulfate–proteoglycan enriched anionic sites of glomerular basement membrane and mesangial matrix were studied. Rat kidneys were treated either with buffers alone or buffers containing heparitinase or chondroitinase-ABC followed by perfusion with cationic or native immune complexes. Tissues were processed for immunofluorescence and transmission electron microscopy after fixation with glutaraldehyde or tannic acid glutaraldehyde. Kidneys perfused with radioiodinated immune complexes were processed for light and electron microscopic autoradiography. In addition, glomeruli from kidneys perfused with radioiodinated immune complexes were isolated and counted for radioactivity. By immunofluorescence the cationic immune complexes deposited linearly along the glomerular basement membrane. By electron microscopy, the cationic complexes localized mainly in the inner and outer layers of the glomerular basement membrane and to a certain extent in the mesangial matrix in a distribution that corresponded to previously documented anionic sites. Whereas heparitinase treatment abrogated the binding of cationic immune complexes in both glomerular basement membrane and mesangial matrix, chondroitinase-ABC treatment did not cause any decrease in binding. In contrast, more neutral immune complexes appeared to be nonspecifically trapped in the mesangium, and their distribution was unaffected by both enzymatic treatments. Light and electron microscopic autoradiography and counts of isolated glomeruli confirmed these findings. The results overall indicate that cationic immune complexes bind electrostatically to the heparan sulfate–proteoglycan enriched anionic sites of the glomerular basement membrane and mesangial matrix, while more neutral immune complexes are nonspecifically trapped in the mesangium of the renal glomerulus.

Since the discovery of fixed anionic sites [1–4] in the basement membrane (GBM)[1] of the renal glomerular capillary wall, the notion that these sites may be responsible for trapping immune complexes or in situ immune complex formation has attracted the attention of several investigators [5–15]. Evidence that these sites are indeed involved has been gradually accumulating. It is more or less established that cationic substances, including immune complexes as well as free antigens or antibodies, localize in the glomerular capillaries, specifically in the laminae rarae interna and externa of the GBM. On the other hand, more neutral macromolecules, including immune complexes, lodge nonspecifically in the mesangial regions of the glomerulus. Immune complexes with both cationic antigen and antibody have highly accentuated binding to GBM compared to the mesangial matrix, whereas immune complexes with cationic antigen or antibody alone may have somewhat different binding characteristics depending upon the degree of cationization [5]. Thus, it seems that anionic sites play a significant role in the trapping of immune complexes, most likely by ionic interaction with the negatively–charged sulfate or carboxyl groups of some of the acidic glycoproteins of the GBM. At least part of the electronegativity of the anionic sites is due to the sulfate groups of heparan sulfate–proteoglycans (HS-PG) [16, 17]. The HS-PG of the GBM has a molecular weight of ~130,000 with a core protein (mol wt ~18,000) to which 4-5 glycosaminoglycan chains (mol wt ~26,000) are attached [18, 19]. Such a molecular structure for the HS-PG would form a highly intricate meshwork that would also endow the glomerular capillary wall with a very high electronegative charge.

Although the ultrastructural distribution of cationic immune complexes and probes is similar [5, 8], until now it has remained problematic whether the sulfate residues of HS-PG, a part of the anionic sites, are the actual entrapment/binding regions for cationic immune complexes. We have explored this possibility in the present investigation, and the data indicate that the sulfate residues of HS-PG do indeed bind cationic complexes in both glomerular extracellular matrices, primarily the GBM and to some extent the mesangial matrix. On the other hand, HS-PG apparently plays a much less significant role in the nonspecific lodging of neutral immune complexes in the mesangium.

Methods

Antigen and antibody

Two types of antigens were used, either native bovine gamma globulin (BGG; Sigma Chemical Co., St. Louis, Missouri, USA) or cationic BGG. Native BGG was further purified by passage through Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA) in 0.01 M phosphate buffered saline, pH 7.2. The purified fraction was utilized as an antigen for raising antibodies and also for preparing cationic BGG. Rabbits were immunized with BGG in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA), and their antisera obtained and decomplemented by heating at 56°C

[1]Abbreviations used in this paper are: BGG, bovine gamma globulin; GBM, glomerular basement membrane; HS-PG, heparan sulfate–proteoglycans.

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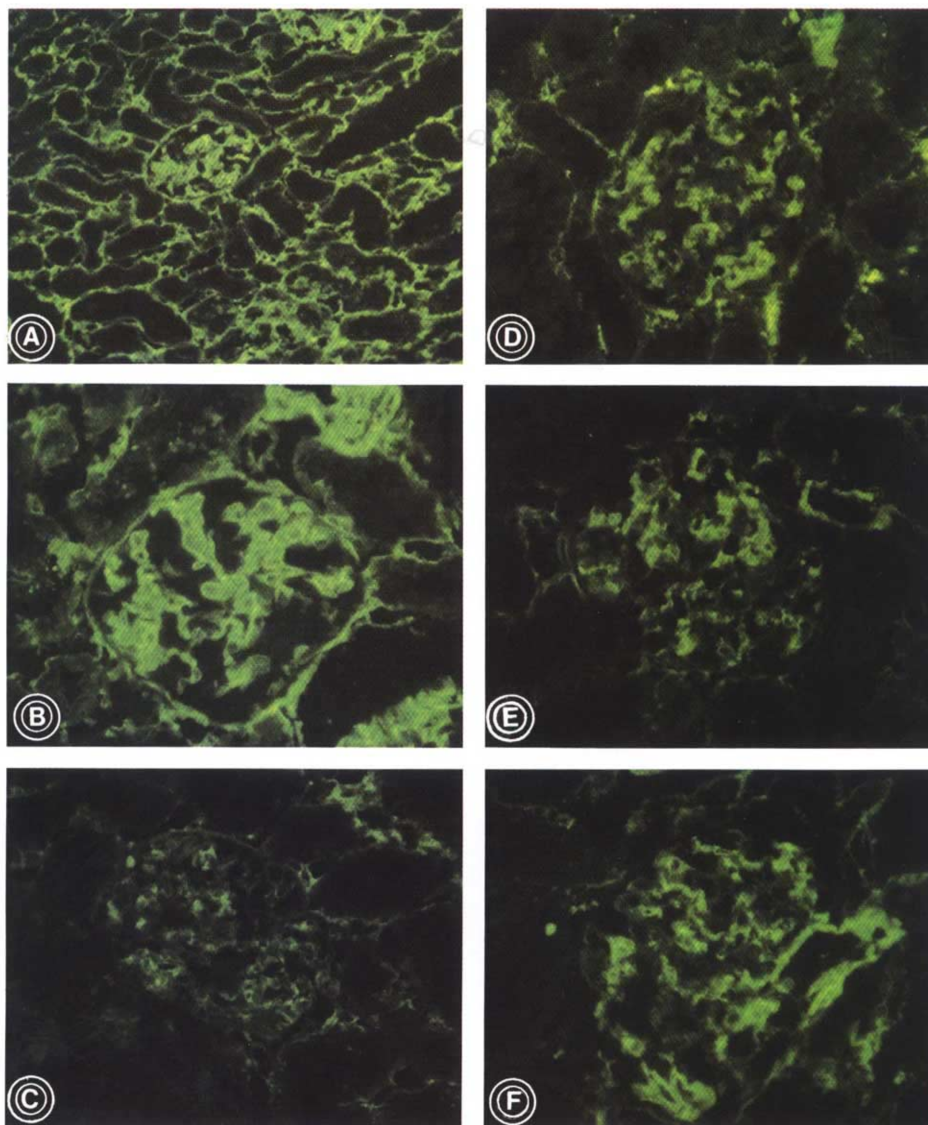

Fig. 1. *Immunofluorescent micrographs of glomeruli from kidneys perfused with cationic (**A**, **B**, and **C**) or native (**D**, **E**, and **F**) immune complexes after treatment with buffer (**A** and **D**), chondroitinase-ABC (**B** and **E**) or heparitinase (**C** and **F**).* Fig. A × 150; Figs. B–F × 400.

for 30 minutes. Anti-BGG IgG fraction was prepared by precipitation in half-saturated ammonium sulfate followed by passage through a diethylaminoethyl (DEAE)-cellulose (Schleicher and Schuell Inc., Keene, New Hampshire, USA) column in 0.0175 M potassium phosphate, pH 6.30 [5]. Cationic BGG was prepared by cationization of BGG by a two-step reaction with ethylenediamine and carbodiimide [5, 20]. The cationic BGG was then extensively dialyzed against normal saline, lyophilized and dissolved in phosphate buffered saline, pH 7.2.

Unlabelled immune complexes

Two types of soluble complexes were prepared as previously described and characterized [5]: (a) native complexes, that is, native heterogeneous BGG combined with native heterogeneous rabbit anti-BGG; and (b) cationic complexes, that is, chemically modified cationic BGG (isoelectric point, pI, 9.5 to 11.5) combined with native heterogeneous rabbit anti-BGG. The rabbit IgG fraction contained 11% of specific antibody directed towards BGG. Based on quantitative immune precipitation to determine the equivalence point [21], immune complexes were prepared in fivefold antigen excess at room temperature, pH 7.2. Complexes were left overnight at 4°C and centrifuged at 10,000 ×g for 10 minutes to remove any aggregates before use. These differently charged complexes, which are not distinguishable by size, extend over the range 9 to 25S by sedimentation velocity [5].

Radioiodinated immune complexes

For preparation of radioiodinated immune complexes the rabbit anti-BGG was conjugated with ^{125}I-iodine (Amersham Co., Arlington Heights, Illinois, USA) by the lactoperoxidase–glucose oxidase method [22, 23]. The iodinated product was extensively dialyzed against normal saline to remove free iodine and the protein concentration calculated by $E_{280}{}^{1\%} = 14$. Complexes were formed in the same manner as above. To prepare cationic complexes, 59 mg of radioactive rabbit anti-BGG containing 6.5 mg of specific antibody was added to 3.1

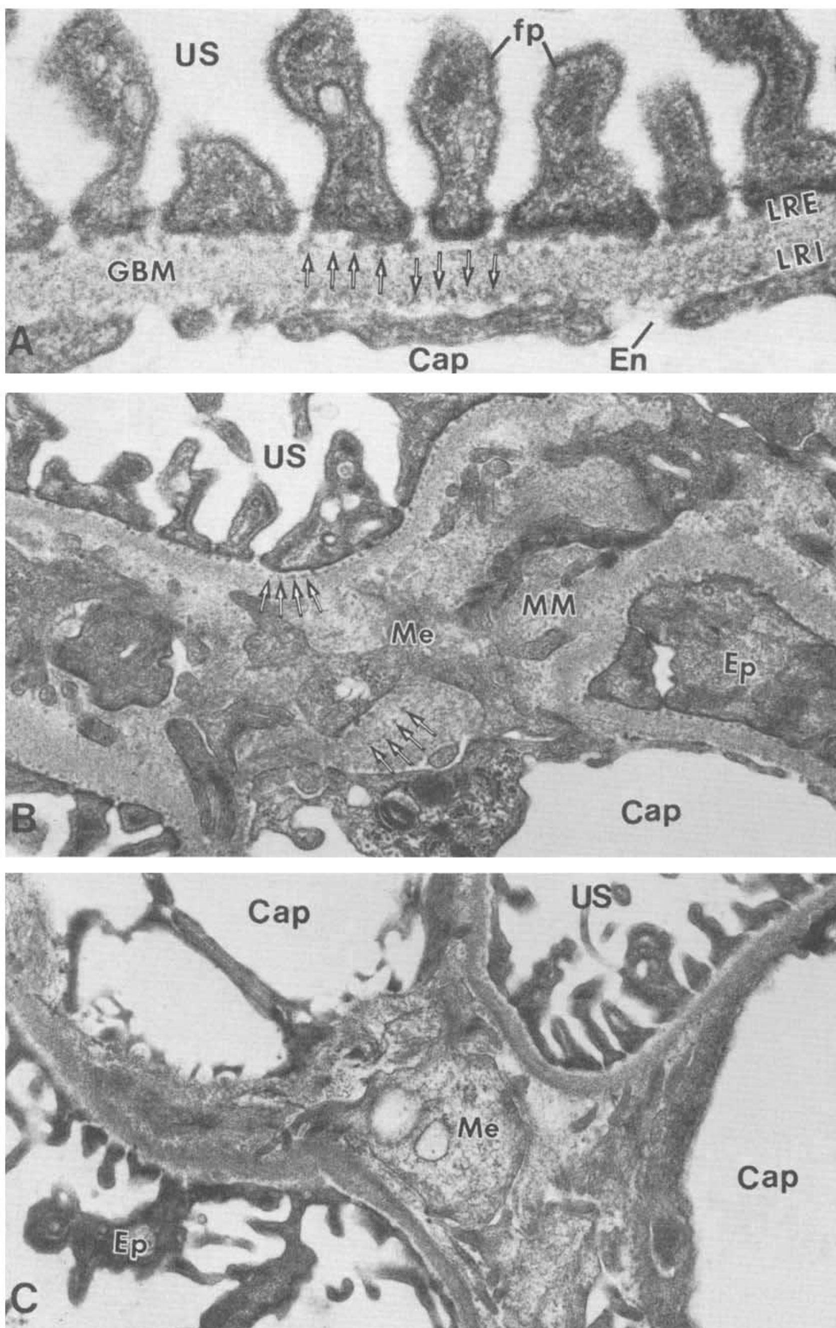

Fig. 2. *Electron micrographs of glomeruli from kidneys perfused with cationic immune complexes after treatment with buffer* (**A**), *chondroitinase-ABC* (**B**) *or heparitinase* (**C**). Characteristically, cationic immune complexes are seen as electron dense deposits in the laminae rarae interna (LRI) and externa (LRE) of the glomerular basement membrane (GBM) and mesangial matrix (MM) as indicated by arrows (A, B). After treatment with heparitinase (C) the immune complexes no longer bind to the anionic sites. Abbreviations are: fp, foot processes; US, urinary space; En, endothelium; Cap, capillary lumen; Ep, epithelium; Me, mesangium. A × 60,000; B × 15,000; C × 10,000.

mg of cationic BGG with constant stirring at room temperature. For preparing more neutral heterogeneous complexes, 67 mg of radioactive rabbit anti-BGG containing 7.4 mg of specific antibody was added to 2.2 mg of native BGG. The radioiodinated complexes were centrifuged at 10,000 ×g for 10 minutes to remove any aggregates.

Buffer and enzyme solutions

Approximately 1.0 mg of immune complexes, cationic or native, was dissolved in 100 ml of Krebs Ringer bicarbonate buffer containing 7.5% bovine serum albumin. This solution of immune complexes was used for binding studies in kidneys that had been treated with glycosaminoglycan–degrading enzymes. The enzyme solutions included chondroitinase-ABC (Miles Laboratories, Naperville, Illinois, USA, 1.0 unit/ml in Krebs Ringer bicarbonate, pH 7.4) or purified protease–free heparitinase [24, 25] (0.1 mg/ml in Krebs Ringer bicarbonate containing 0.01 M acetate, pH 7.0). The perfusion fixative solution included 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, either alone or containing 0.2% tannic acid to enhance the visualization of immune complexes in the glomerular extracellular matrices.

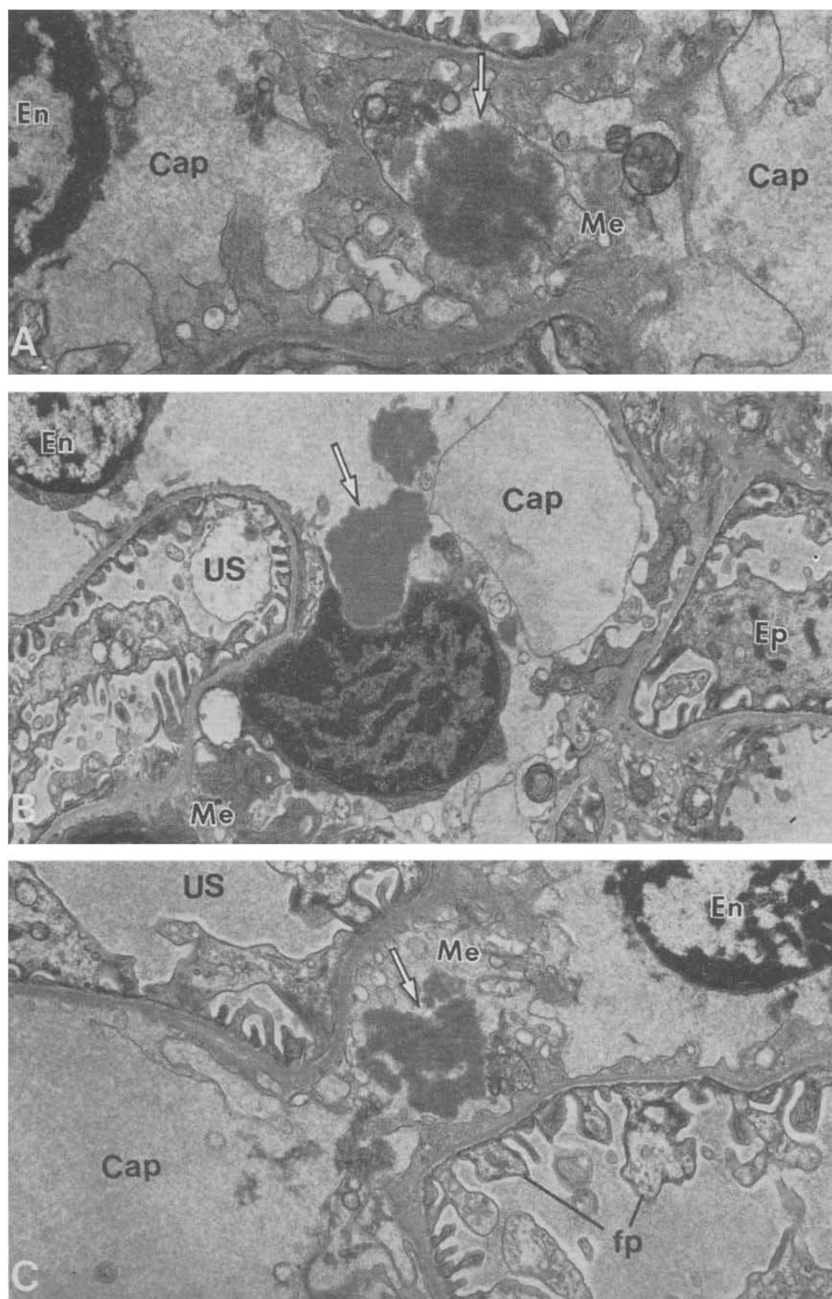

Fig. 3. *Electron micrographs of glomeruli from kidneys perfused with native complexes after treatment with buffer (A), chondroitinase-ABC (B) or heparitinase (C).* The complexes are seen as large electron-dense deposits (arrows) nonspecifically lodged in the mesangium, and their localization is unaffected by the enzymatic treatments. Abbreviations are the same as Fig. 2 (× 12000).

Perfusion with immune complexes

A total of 112 rat kidneys (right) were perfused and utilized for four sets of studies: immunofluorescence, electron microscopy, light and electron microscopic autoradiography, and direct radiocounting of isolated glomeruli. Each set of studies included 16 kidneys with four kidneys per variable, the four variables being the two enzymes and their respective buffer controls. The right renal artery was cannulated and the kidney isolated from the systemic circulation. The kidney was then flushed in situ with Krebs–Ringer bicarbonate albumin solution followed by perfusion with chondroitinase-ABC (degrades chondroitin sulfates ABC and hyaluronic acid) or heparitinase (degrades heparan sulfate), or the corresponding oxygenated control buffer solution at 37°C for 10 minutes. The kidneys were then removed from the abdominal cavity, connected to an organ perfusion system [26] and perfused with oxygenated Krebs–Ringer bicarbonate albumin solution containing either cationic or native immune complexes at 37°C for 20 minutes following which the kidneys were flushed with 40 ml Krebs Ringer bicarbonate albumin solution. Under these conditions, heparitinase degrades the anionic sites of the GBM [25–27], and viability is maintained by the criteria of retention of morphological integrity of glomerular structures and the ability to incorporate sulfate into macromolecules in a linear fashion over time [18, 19, 25].

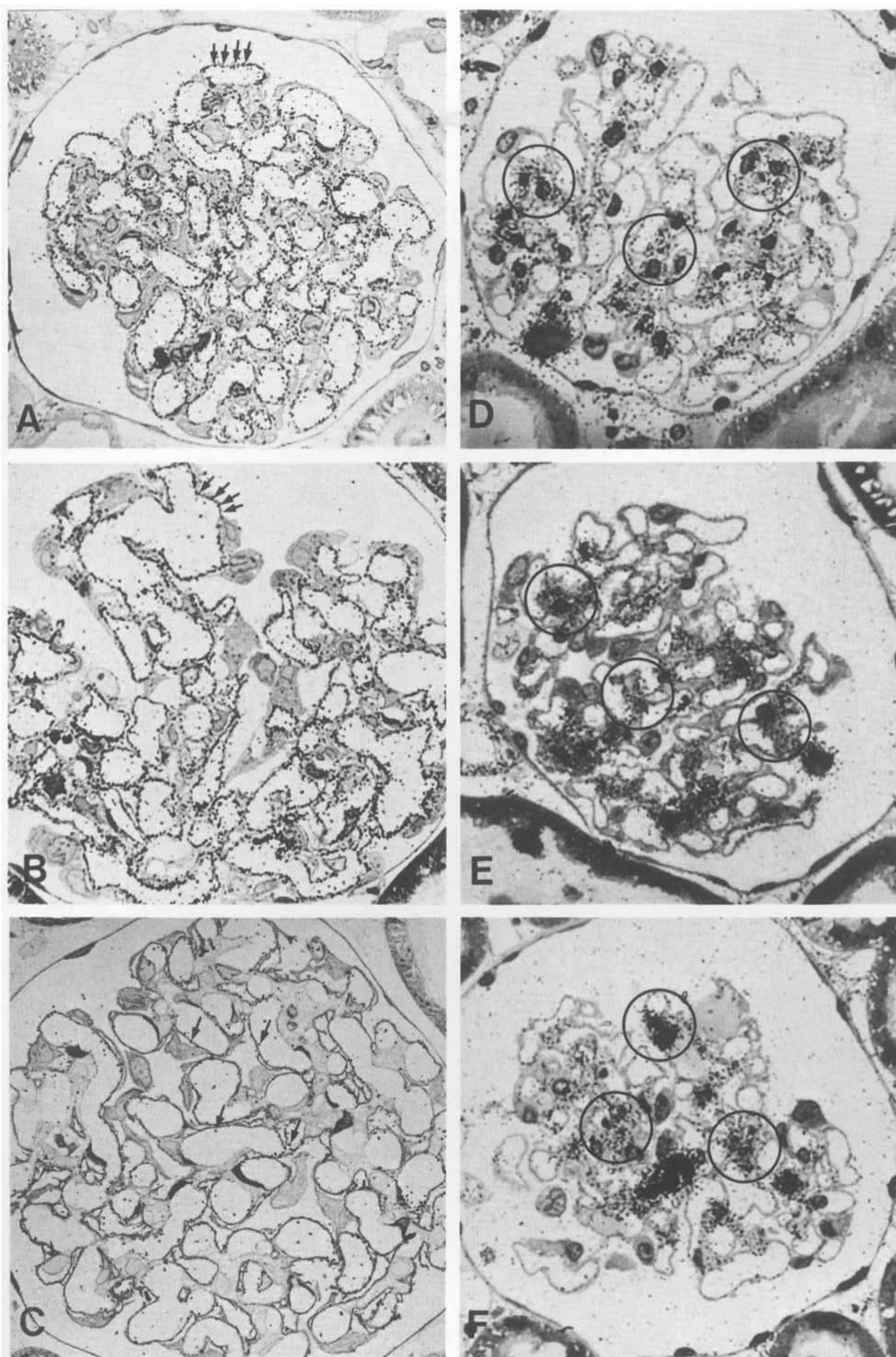

Fig. 4. *Light microscopic autoradiograms of glomeruli obtained from kidneys perfused with ^{125}I-labelled cationic* (**A,B,C**) *or native* (**D,E,F**) *complexes after treatment with buffer (A and D), chondroitinase-ABC (B and E) or heparitinase (C and F).* Arrows indicate the localization of cationic complexes in the capillary walls (A,B) while circles indicate their presence in the mesangium (D–F). Note the marked decrement in cationic complexes after heparitinase (C). × 1,000.

Characterization of binding of immune complexes

For immunofluorescence studies kidney slices were snap frozen in chilled isopentane, 4 mμ cryostat sections made, stained with fluoresceinated anti-rabbit IgG or anti-bovine IgG and examined with an immunofluorescence microscope equipped with epiillumination. For electron microscopic studies the kidneys were fixed by perfusion with tannic acid–glutaraldehyde and processed [26].

For tissue autoradiography, 1 mm^3 cortical pieces were cut from perfused–fixed kidneys, osmicated, treated *en bloc* with uranyl acetate, dehydrated, and embedded in EPON. Thick (0.5 mμ) and thin (600 Å) sections were made and processed for light and quantitative electron microscopic autoradiography [26, 27]. For quantitative autoradiography, 25 glomeruli from each group were photographed at a magnification of × 600 and printed to a final magnification of × 3,000. Capillary and mesangial compartments were defined, the grains associated with each com-

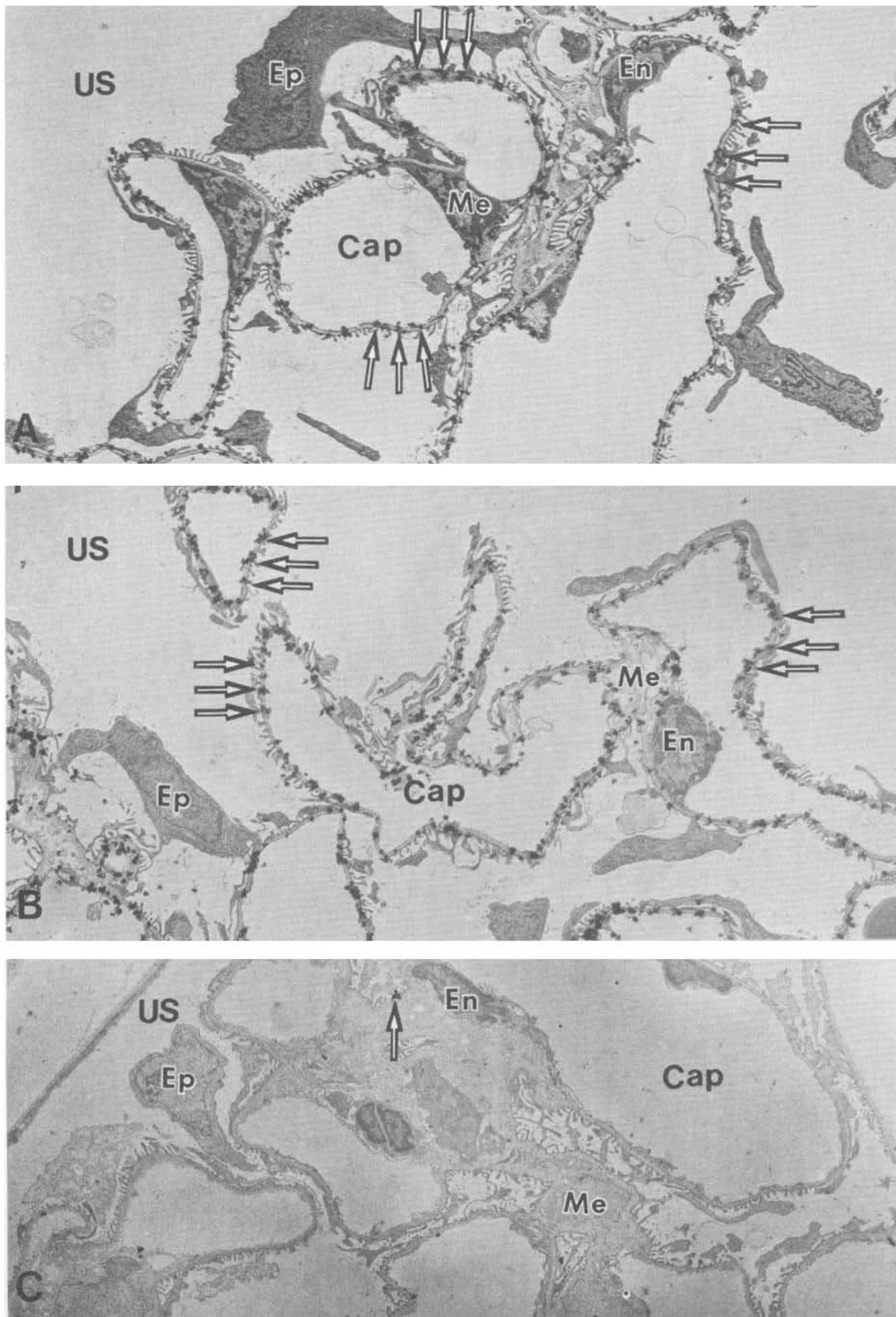

Fig. 5. *Electron microscopic autoradiograms of glomeruli from kidneys perfused with* ^{125}*I-labelled cationic complexes after treatment with buffer* (**A**), *chondroitinase-ABC* (**B**) *or heparitinase* (**C**). The majority of silver grains are on the glomerular capillary walls (arrows); they are diminished after treatment with heparitinase (C). Abbreviations are the same as Fig. 2. × 3,000.

partment were counted and the total area was computed by the point counting method [27], utilizing a transparent overlay network of points spaced 0.5 cm apart. The grain density was computed by dividing total number of grains by total area points. Finally, the mean grain density and standard deviation were calculated.

For radiocounting of glomerular bound ^{125}I-immune complexes, two kidneys for each variable (above) were perfused and subsequently flushed with Krebs–Ringer bicarbonate albumin solution to remove unbound radioactivity. The cortices were dissected free and frozen at −20°C overnight. The glomeruli were then isolated by the sieving method [18, 26], and radioactivity determined by gamma counter and expressed as average counts per kidney.

Results

Immunofluorescent studies revealed similar patterns of staining of sections treated with either fluorescein–conjugated anti-rabbit or anti-bovine IgG, that is, both components of the immune complexes, for both the cationic and native complexes. In control sections from kidneys perfused with cationic complexes, a linear staining corresponding to glomerular and tubular basement membranes was observed (Fig. 1A). Slight mesangial and interstitial staining was also present, and the vascular

Table 1. Grain density distribution of radioiodinated cationic and native immune complexes in different compartments of the glomerulus with and without enzymatic treatments

Treatment	Cationic complexes[a]		Native complexes[a]	
	Capillary wall	Mesangium	Capillary wall	Mesangium
Control	9.30 ± 1.25	0.50 ± 0.11	0.20 ± 0.03	4.34 ± 0.91
Chondroitinase-ABC	9.33 ± 0.96	0.45 ± 0.05	0.20 ± 0.02	4.37 ± 0.48
Control	8.73 ± 1.06	0.48 ± 0.10	0.21 ± 0.02	4.16 ± 0.31
Heparitinase	0.03 ± 0.01	0.02 ± 0.01	0.20 ± 0.01	3.96 ± 0.48

[a] Mean ± SD from 25 glomeruli from each group

Table 2. ^{125}I-associated radioactivity in isolated glomeruli from kidneys perfused with cationic or native immune complexes with and without enzymatic treatments

Treatment	Cationic complexes[a]	Native Complexes[a]
Control	2,100	1,600
Chondroitinase-ABC	2,200	1,400
Control	1,900	1,700
Heparitinase	2	1,600

[a] Each number is the average glomerular counts per minute per kidney obtained from two kidneys (~9,500 glomeruli per kidney) × 10^{-3}

basement membranes evidenced moderate reactivities with the immune complexes as well. Prior chondroitinase-ABC treatment did not change the pattern of immunofluorescent staining (Fig. 1B). Treatment with heparitinase, however, abolished binding of cationic immune complexes (Fig. 1C) as indicated by the lack of immunofluorescence of glomerular and tubular basement membranes. Sections obtained from kidneys perfused with more neutral native immune complexes revealed staining primarily of the mesangium rather than the basement membrane (Fig. 1D). This mesangial staining was unaffected by treatment with chondroitinase-ABC or heparitinase (Fig. 1E, F).

Electron microscopic studies were carried out to determine the precise localization of immune complexes in kidneys fixed by tannic acid–glutaraldehyde perfusion. In a cross–section of the glomerular capillary, the cationic complexes were visualized as electron–dense deposits in the laminae rarae interna and externa of the GBM (Fig. 2A), very much reminiscent of the localization of anionic sites [4]. The deposits were regularly spaced at intervals of 600 Å and exhibited a lattice–like network in grazing sections of the basement membranes. The lattice–like distribution could also be seen in the mesangial matrices as well (Fig. 2B). Treatment with chondroitinase-ABC did not alter the distribution of electron–dense deposits (Fig. 2B), whereas digestion with heparitinase resulted in the loss of binding of immune complexes and non-visualization of deposits in the GBM and mesangial matrix (Fig. 2C). The more neutral native complexes on the other hand seemed to be nonspecifically lodged in the mesangial region, unaffected by the enzymatic treatments, and with no specific relationship to glomerular extracellular matrices (Fig. 3). Some complexes were seen in the process of being phagocytosed by the monocyte–like cells in the mesangium (Fig. 3B).

Tissue autoradiographic studies were performed to quantify the distribution of immune complexes in various regions of the glomerulus and to determine more accurately the enzymatic effects on their localization. Similar to the immunofluorescent findings, by light microscopy the radioiodinated cationic immune complexes had a characteristic capillary distribution (Fig. 4A), unaffected by chondroitinase-ABC treatment (Fig. 4B), but abolished by heparitinase digestion (Fig. 4C). The localization of the native complexes was similar to what was observed in earlier studies, namely mesangial, and unaffected by the enzymes (Fig. 4D–F). Electron microscopic autoradiography showed more precisely the differential localization of the cationic immune complexes in the glomerular capillary wall compared to the mesangium (Fig. 5A), with silver grain densities (concentration of radiation) of 9.30 ± 1.25 and 0.50 ± 0.11, respectively (Table 1). No significant change in the grain density distribution was observed after chondroitinase-ABC treatment (Fig. 5B and Table 1), whereas heparitinase digestion resulted in a remarkable reduction in both the glomerular capillary wall and mesangium (Fig. 5C); grain densities were 0.03 ± 0.01 and 0.02 ± 0.01, respectively (Table 1). The grain density distributions in kidneys perfused with native complexes were 0.20 ± 0.03 and 4.34 ± 0.91 (chondroitinase buffer control) in the capillary wall and mesangium, respectively, and no statistically significant change was observed after enzymatic treatments (Table 1 and Fig. 6).

Further confirmation of the binding characteristics of the immune complexes was carried out by isolating glomeruli and directly determining their radioactivity. Similar trends as observed by immunofluorescence, electron microscopy, and tissue autoradiography were noted (Table 2). The significant result was the thousandfold reduction in binding of cationic complexes after heparitinase treatment.

Discussion

Via immunofluorescent, electron microscopic, autoradiographic, and direct counting techniques, converging evidence has been obtained in this investigation that proteoglycans, that is HS-PG, known to be integral components of glomerular anionic sites, play a definitive role in the binding/deposition of immune complexes with cationic charge. In contrast, the proteoglycans do not seem to play a significant role in trapping more neutral complexes. The multiple approaches were used in an isolated organ perfusion system to arrive at concrete conclusions, which in previous investigations were somewhat conjectural.

An ex vivo system was needed for a variety of reasons. Firstly, the potential problem of in vivo dissociation of complexes can be obviated adequately. Secondly, in this system trapping/binding of immune complexes is studied independent of humoral factors, such as complement. Thirdly, to ascertain the role of proteoglycans in the binding of immune complexes, molecular alterations have to be made in the proteoglycans. This can be achieved in two ways, either by enzymatic digestion whereby the glycosaminoglycan chains are degraded with consequent disruption of the network of these highly charged polymers [24], or by xyloside treatment, in which case the carbohydrate chains are not transferred properly by the xylosyltransferase for covalent attachment to the core protein

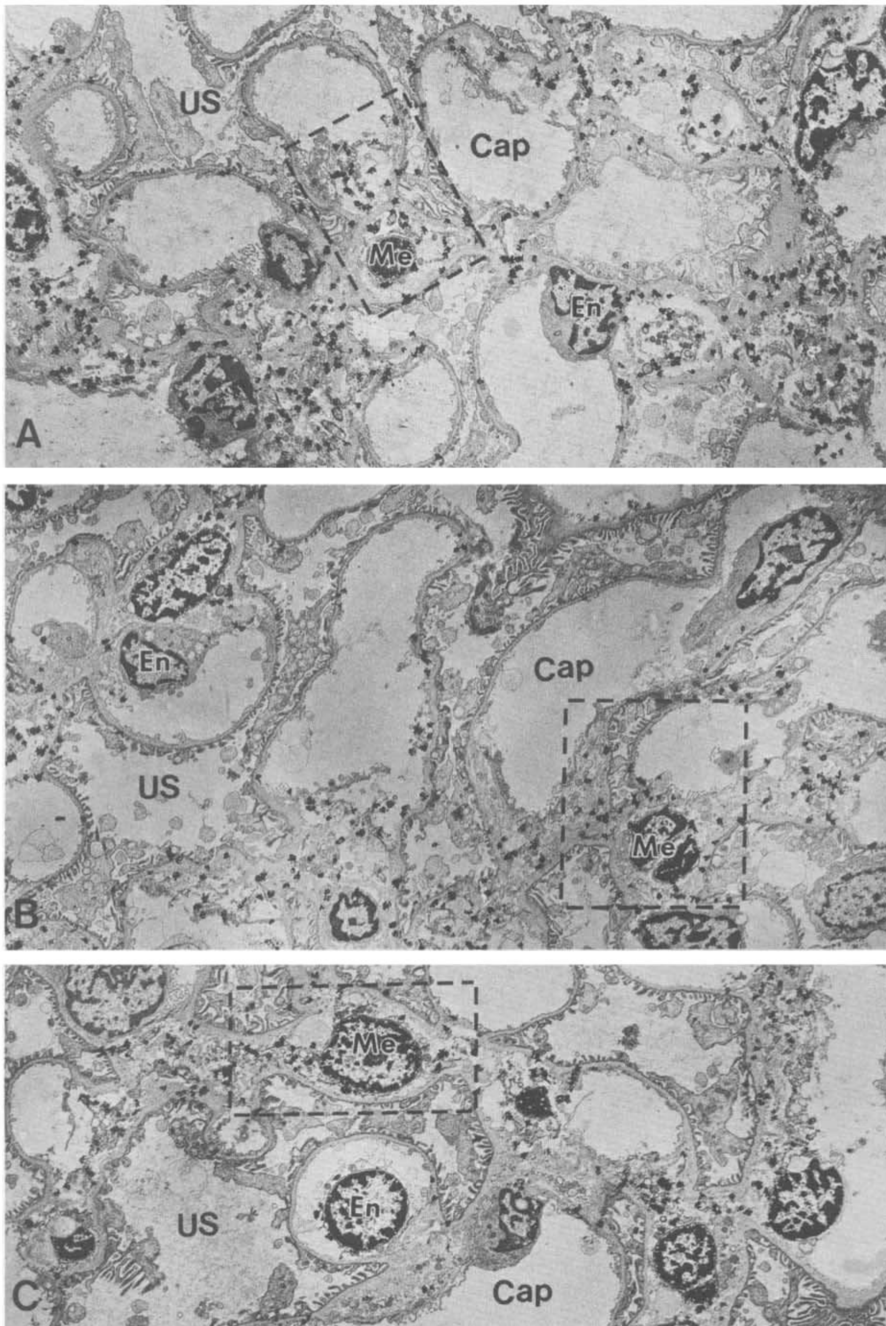

Fig. 6. *Electron microscopic autoradiograms of glomeruli from kidneys perfused with ^{125}I-labelled native complexes after treatment with buffer* (**A**), *chondroitinase-ABC* (**B**) *or heparitinase* (**C**). The majority of the silver grains are in the mesangium and their distribution is unaffected by the enzymatic treatments. Abbreviations are the same as Fig. 2 (× 3,000).

with resultant abortive organization of the HS-PG molecule [28]. Such xyloside treatment is ineffective in in vivo systems because of its metabolic degradation, and weeks are required to achieve alterations in the HS-PG macromolecular complex in ex vivo and in vitro states. Hence, to address the issues of the present investigation it was decided to employ enzymatic treatment with ex vivo organ perfusion, a system that does not seem to compromise the results obtained and conclusions drawn in this or past investigations.

Proteoglycans, existing in extracellular matrices as large macromolecules with lateral and/or end-to-end aggregation, form a gel meshwork which is kept highly hydrated by water molecules trapped in the interstices of the matrix [16, 29–31]. This matrix linked to other basement membrane components is thought to give the GBM a viscous, gel-like consistency [32]. Such organization would promote or deter the binding of immune complexes largely on an electrostatic basis and to a lesser extent by virtue of steric entrapment. However, both these effects of proteoglycans are more or less interwoven and there is no ready means to study separately their roles in the deposition of immune complexes. Nevertheless, the fact that quantitative autoradiographic data indicate a negligible trapping of more neutral complexes but exclusive deposition of cationic complexes in the capillary wall or GBM suggests that it is

mainly coulombic forces which are involved, in agreement with previous work showing that intact immune complexes of molecular weight greater than one million can penetrate the GBM if sufficiently cationic [8].

The available evidence thus strongly suggests that if immune complexes carry sufficient positive charge, whether due to antigen, antibody or both, they will bind to the sulfate groups of the proteoglycans. In particular, the data presented here provide direct evidence that these negatively charged residues of HS-PG are involved in such interactions. In previous studies the significance of the net charge of the complex and the respective contributions of antigen and antibody have been debated. Nevertheless, the results obtained here reinforce the concept that the charge of individual components of the complex is less significant and that the net charge of the complex as a whole is the important determinant of sites of reaction within the glomerulus. These considerations are valid as long as the immune complexes are not highly dissociable, and are soluble and thus more easily diffusible. Previous results from our laboratories [5, 8] indicate that the BGG-anti-BGG complexes we have used, whether covalently linked or otherwise, have identical distributions when administered intravenously. Similarly, the fact that in the current studies the same unmodified antibody was common to both the cationic and the more neutral complexes and yet localized in different compartments of the glomerulus argues that the complexes did not significantly dissociate under the conditions used, and that it was the antigens which ultimately influenced the net charge of the complexes and their resultant patterns of deposition.

It now seems clear that immune complexes may entangle and precipitate in a meshwork of proteoglycans primarily via electrostatic forces. The present data certainly support such a contention, originally proposed by Hellsing [33, 34]. The degree to which these concepts apply to proteoglycans in human kidneys and to human forms of immune complex nephropathy remains to be determined. In this regard, it may be worth considering that in the immune complexes employed in our model system, the antibody was in its native state, and the range of isoelectric points represented in the cationized BGG antigen was comparable to that of a number of common proteins including lysozyme, cytochrome C, histones, and protamines, all of which have isoelectric points between 9.8 and 12.1 [35]. It should also be kept in mind that because immune complexes are heterogeneous, circulating complexes may not be representative of the fraction which is most prone to cause disease; in fact, it is possible that the complexes with the greatest affinity for the GBM deposit so quickly that their detection in serum becomes problematic.

Acknowledgments

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