ENHANCEMENT OF GLOMERULAR IMMUNE COMPLEX DEPOSITION BY A CIRCULATING POLYCATION

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The net charge of antigens, antibodies, or of immune complexes may affect their localization in glomeruli (1–3). A positive net charge favors increased localization in the glomerular basement membrane (GBM),[1] whereas a negative net charge favors mesangial deposits (1–3). These studies examined the interaction of differently charged immune reactants with polyanions[2] in normal glomeruli.

Conversely, perturbations of glomerular polyanions may alter glomerular interactions with immune reactants and affect their localization. Hypothetically, secretory cationic proteins from activated leukocytes and platelets may bind to glomeruli during the earliest stages of immune injury and enhance glomerular permeability, favoring immune complex deposition. Circulating exogenously derived polycations bind to polyanions in the GBM and increase permeability to proteins (6–9). Here, we show that administration of the polycation polyethyleneimine (PEI), which is known to bind to the GBM in vivo (10) markedly enhances the deposition of preformed immune complexes in glomeruli, particularly in the GBM.

Methods

Immunizations. New Zealand White rabbits were immunized against bovine serum albumin (BSA) Fraction V (11). Sera were assayed by immunodiffusion, and the strongest antisera were pooled. Pooled serum was fractionated by precipitation with 18% saturated sodium sulfate, and dialyzed against 0.02 M phosphate-buffered saline, pH 7.35 (PBS). Antibody was assayed by quantitative immunoprecipitin analysis (12). Isoelectric focusing of the immunoglobulin fractions on slab gels of 5.0% acrylamide and 1.0% *bis*-acrylamide containing 2.5% ampholine (pH range 3.5–10.0) revealed multiple bands ranging in isoelectric points (pI) between 5.3 to 8.0. The pI of BSA was determined to be 4.9–5.1.

Preparation of Immune Complexes. Soluble immune complexes were prepared as described by Germuth et al. (13). The equivalence amount of BSA was added to pooled

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[1] *Abbreviations used in the paper:* BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; PBS, phosphate-buffered saline; PEI, polyethyleneimine.

[2] Connective tissue elements with a net negative charge are known to be present in the cell surface coats of glomerular capillary endothelial cells and epithelial cells, and in the GBM. Such polyanions, which may be sialoproteins, protein carboxyls, and sulfated proteoglycans, are thought to play major roles in many aspects of glomerular structure and function. Polyanions in the GBM, in particular, appear to determine charge selective interactions of the glomerular capillary wall with macromolecules (4, 5).

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fractions, mixed, and incubated at 37°C for 1 h and 4°C overnight. The precipitate was washed three times with PBS, then suspended in a BSA solution to make soluble immune complexes at 40 times antigen excess and incubated at 37°C for 1 h and 4°C overnight. Before injection into rats, the immune complex solution was centrifuged at 48,000 *g* for 60 min and sterile filtered. In additional studies, affinity-purified anti-BSA was labeled with ^{125}I (Iodine) and then added to the anti-BSA immunoglobulin fraction before preparation of immune complexes as outlined above.

Polycation. PEI, 18 (mol wt 1,800), obtained from Arsynco, Inc. (Carlstadt, NJ), was diluted in 0.05 M PBS, pH 7.2, to make a final solution containing 0.5% PEI with a pH of 7.4. In control experiments, 0.05 M PBS, pH 7.4 without PEI was used.

Protocol. 12 male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI), weighing 200–290 g, were paired according to age and size, then divided into two groups. Rats from Group I were briefly anesthetized with ether and then injected with PEI at a dose of 10 μg/g body weight via a tail vein. PEI administered in this manner binds to polyanions present in the GBM and enhances glomerular permeability to the tracer macromolecule ferritin (molecular radius = 61 Å (8). 5 min later, ~1 ml of preformed immune complexes containing 30 mg of anti-BSA antibody in 40 times antigen excess was injected intravenously. Controls (Group II) received PBS without PEI followed by the same dose of immune complexes as described above. To assess any toxic effect of PEI, an additional four rats (Group III) were given PEI, as outlined above, but without subsequent injection of immune complexes. In each group, all injections were repeated two additional times 4 h apart. After each series of injections, the rats were placed in metabolic cages and urine collected for subsequent protein analysis. 1 h after the last injection, the rats were anesthetized, the left renal pedicle ligated, and the left kidney excised, sliced, and frozen in chilled isopentane. Immediately following removal of the left kidney, the right kidney was fixed by perfusion with 1.25% glutaraldehyde in cacodylate buffer, pH 7.4, and subsequently processed for light and electron microscopy using routine methods. In an additional study, the ultrastructural localization of immune deposits was assessed by indirect immunoperoxidase procedures to reveal rabbit IgG. Immune reactions were performed on 60-μm tissue sections and exposure time to 3,3′-diaminobenzidine was kept to a minimum (1 min) as recommended by Courtoy et al. (14).

Glomerular localization of BSA and rabbit IgG was assessed in sections of renal cortex by direct immunofluorescence, using fluorescein isothiocyanate (FITC)-conjugated IgG fractions of appropriate antiserum. Frozen sections (8-μm thick) were viewed and photographed using a Zeiss Universal II Research microscope equipped with epifluorescence and using a standard FITC filter set supplemented with a KP 560 shortwave pass filter. The intensity of glomerular immunofluorescence of localized BSA and rabbit IgG was quantitated by photometric analysis, using a Zeiss photometer attachment SF connected to a PMI-2 indicator. Intensity of fluorescence in a 125-μm field (approximate size of glomeruli) was obtained by using a 2.5-mm aperture in the light path between the specimen and the photometer. Fluorescence intensity of glomeruli was determined as the relative percent transmission compared to a fluorescein filter standard. All comparisons were expressed as the ratio of fluorescence intensity between PEI (Group I) and control (Group II) in each paired experiment. At least 25 glomeruli were examined in each tissue section.

In experiments in which radiolabeled immune complexes were used, blood samples were collected 5 min before the third injection of PEI or PBS and at termination of the experiment, to determine the serum concentrations of ^{125}I-immune complexes. The size distribution of immune complexes in serum samples was determined by sucrose density ultracentrifugation. 200 μl of serum was applied to sucrose gradients (10–40%), followed by centrifugation at 100,000 *g* for 16 h. Size distribution of immune complexes was determined by continuous spectrophotometric measurements at 280 nm and by isotopic analysis of 15-drop fractions.

Results

Administration of PEI enhanced the deposition and altered the distribution of glomerular deposits as determined by electron microscopy. All capillary walls

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from all glomeruli examined of Group I, showed numerous small electron-dense deposits in the subendothelium and to a lesser extent in the subepithelium (Fig. 1 *a*). Peripheral capillary wall deposits were absent in glomeruli of Group II (controls) (Fig 1 *b*). The mesangial matrix of both groups showed infrequent dense deposits by electron microscopy. Deposits were more numerous in Group I rats than in controls (Group II). Immunoperoxidase methodology revealed the localization of rabbit IgG identical in distribution to that of the dense deposits observed by routine staining techniques, substantiating their immune nature (Figs. 2 *a* and *b*).

Hypercellularity or inflammatory cell infiltrates were not observed in any of the three groups. Some glomeruli from Group I showed luminal strands of fibrin and platelets; however, this observation was focal and most capillary loops were patent and free of luminal material. Glomeruli of controls (Group II) did not show fibrin or platelets in capillary lumina. Similarly, administration of PEI alone without subsequent injection of immune complexes (Group III) did not result in glomerular alterations.

Enhancement of glomerular localization of immune complexes by PEI was verified by quantitative immunofluorescence. Glomeruli of Group I rats showed substantially greater intensity of fluorescence of localized rabbit IgG and BSA compared with paired controls (Group II) (Table I). Rabbit IgG and BSA localized within the mesangium of both groups (Figs. 3 *a* and *c*). However, localization of IgG was much more diffuse in Group I rats and also involved some peripheral capillary loops (Fig. 3 *a*). Despite localization of immune deposits

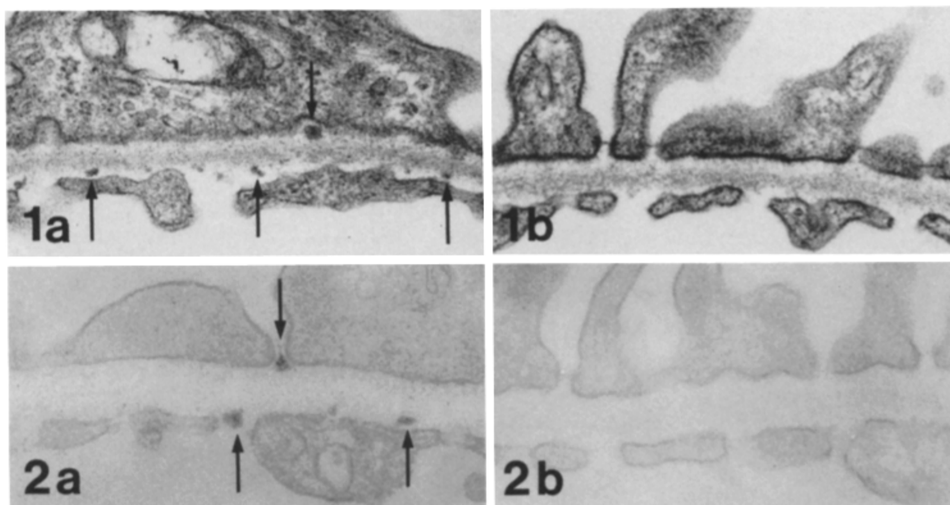

FIGURE 1. Electron micrographs of glomerular capillary walls of (*a*) Group I (PEI plus immune complexes) and (*b*) Group II (control, diluent plus immune complexes). Electron-dense deposits (arrows) are localized in the subendothelial and subepithelial aspects of the GBM of Group I (*a*), but absent in the GBM of Group II (*b*). × 37,000.

FIGURE 2. Glomerular localization of rabbit IgG by immunoperoxidase electron microscopy. In glomeruli of Group I (*a*), reaction product (arrows) is localized in an identical distribution as the dense deposits observed by routine staining methods (see Fig. 1 *a*). Reaction product (rabbit IgG) is absent in the GBM of Group II controls (*b*). × 49,000.

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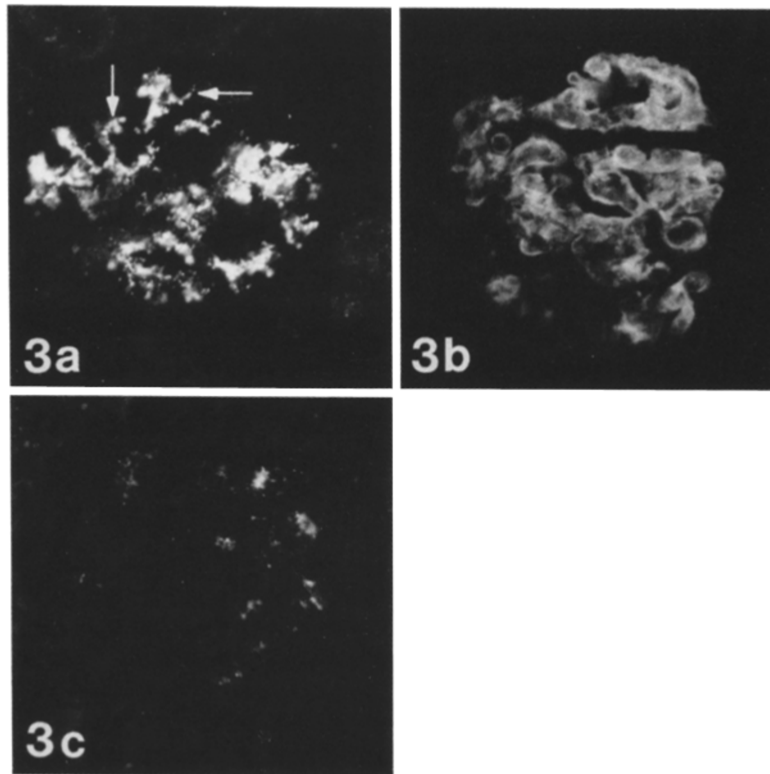

FIGURE 3. Glomerular immunofluorescence localization of rabbit IgG of Group I (*a* and *b*) and Group II (*c*) rats. In Group I, staining is predominantly mesangial but some peripheral capillary loops (arrows) also show IgG localization (*a*). In some rats localization of rabbit IgG was observed in peripheral capillary loops (*b*). Group II controls showed only mesangial localization of rabbit IgG in a segmental distribution (*c*). × 340.

in the GBM of all peripheral capillary loops by electron microscopy (Figs. 1 *a* and 2 *a*), IgG and BSA were not always detected by immunofluorescence in this location, most likely reflecting the lower sensitivity of light microscopic techniques. However, in two Group I rats, rabbit IgG and BSA were also observed in a linear pattern in glomerular capillary loops (Fig. 3 *b*). In all cases, localization of rabbit IgG and BSA was absent in peripheral capillary loops of glomeruli from Group II rats, corresponding to the electron microscopic observations.

Serum concentration of ^{125}I-anti BSA-BSA immune complexes at the first collection period was not significantly different between Groups I and II (Group I = 2.59 ± 0.25 (SEM) mg/ml vs. Group II = 2.12 ± 0.25 mg/ml ($P < 0.20$). However, serum concentration of immune complexes in blood samples taken at the termination of the experiment was significantly greater in Group I (4.50 ± 0.27 mg/ml) when compared with Group II controls (3.66 ± 0.17 mg/ml, $P < 0.05$).

To rule out serum concentration of circulating immune complexes as a factor in the enhancement of glomerular localization of deposits in Group I, the dose of immune complexes administered was adjusted to match serum concentrations

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observed in Group II. In these studies, the dose of immune complexes administered to PEI-treated rats was reduced by 30% (the maximum difference observed between Group I and Group II). Reduction of the dose of immune complexes administered to Group I rats lowered serum levels of immune complexes to the same level as Group II but had no effect on the difference in localization and distribution of immune deposits as examined by immunofluorescence (Table I) and electron microscopy.

Continuous spectrophotometric analysis of the effluent of the sucrose gradients after centrifugation showed parallel patterns of protein distribution in serum samples of both groups, indicating that the size distribution of immune complexes in serum was the same in both groups, and that PEI did not interfere with antibody-antigen equilibrium in vivo. Isotopic analysis of 15-drop fractions also showed a parallel distribution of ^{125}I-immune complexes. In all serum samples immune complexes were heterogenous in size; however, the peak counts occurred in Fractions 5–7 (corresponding to thyroglobulin standard with a molecular weight of 669,000 daltons).

Urine protein excretion of Group I rats was significantly higher than that measured in Group II rats (Group I = 37.2 ± 18.6 mg; Group II = 10.2 ± 3.3 mg) over the 9-h course of the experiments. Protein excretion of Group III rats was 2.21 ± 0.44/9 h. Hemoglobin was observed in the urine of Group I rats, but absent in Groups II and III and, in part, contributed to the difference in urine protein levels between groups.

TABLE I
*Intensity of Glomerular Immunofluorescence of Localized Rabbit IgG and BSA**

% Transmittance—Rabbit IgG‡			% Transmittance—BSA‡		
Group I	Group II	Group I/ Group II	Group I	Group II	Group I/ Group II
45.7	26.6	1.7	ND	ND	—
49.2	9.2	5.3	55.4	7.9	7.0
28.2	11.2	2.5	20.8	11.1	1.9
42.9	2.4	17.9	56.0	1.9	29.5
48.4	6.3	7.7	38.9	15.7	2.5
39.6	10.7	3.7	40.0	17.5	2.3
34.7[§]	8.4	4.1	19.0[§]	7.9	2.4
30.5[§]	10.7	2.9	38.8[§]	17.5	2.2

Group I, polyethyleneimine plus immune complexes; Group II, diluent plus immune complexes. *ND*, not detected.

* Immunofluorescence localization is primarily mesangial. Electron microscopy revealed additional immune deposits in all peripheral capillary loops of Group I. Glomerular capillary loop immune deposits were absent in Group II (see Figs. 1 and 2). Values are means of at least 25 measurements per kidney.

‡ Percent transmittance of fluorescence intensity compared to a FITC filter standard.

§ Dose of immune complexes adjusted to match serum levels in Group II.

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Discussion

Negatively charged elements in glomeruli constitute an electrostatic barrier to circulating anionic macromolecules (15). PEI is known to bind to polyanions in the glomerular capillary wall, particularly the GBM, in vitro and in vivo (10). Previous studies in our laboratory have shown that PEI dramatically increases glomerular permeability to anionic and cationic ferritins, indicating perturbations in both charge and size selective permeability barriers to circulating macromolecules (8). This study shows that PEI also enhances the deposition and alters the distribution of glomerular immune deposits. In Group I rats (PEI plus immune complexes) immune deposits were observed within the GBM in all capillary loops and in the mesangium. However, in Group II (controls), deposits were absent in peripheral capillary walls but observed exclusively within the mesangium in a segmental distribution in amounts smaller than Group I.

The serum concentration of circulating immune complexes in rats given PEI (Group I) was modestly higher than in controls given diluent alone (Group II). The reason for the difference is not known, but may be related to perturbation of clearance mechanisms. However, the difference in glomerular immune complex localization between the two groups cannot be attributed to this factor. Adjustment of the dose given to Group I to match the blood levels of complexes in Group II did not change the magnitude or the distribution of glomerular immune deposits. Thus, a direct effect of PEI on glomerular permeability to immune complexes is suggested.

The possibility that circulating polycations cause the release of vasoactive amines from leukocytes and/or platelets and secondarily induce increased glomerular permeability may be considered in this context. However, administration of antihistamine and antiserotonin drugs does not diminish polycation-induced glomerular permeability changes (6, 8). Also, polycations induce marked increases of glomerular permeability in perfused kidneys, in the absence of blood and systemic factors (9). These considerations suggest that the presently demonstrated action of PEI depends on direct interaction with the GBM.

The present study does not address the mode in which immune complexes may deposit within glomerular structures (trapping of intact immune complexes vs. formation of deposits in situ). The protocol used in this study (administration of *preformed* immune complexes) would favor the former mode, although recombination of dissociated immune reactants in situ cannot be ruled out. PEI did not alter the size distribution of immune complexes in vivo, suggesting that the equilibrium between immune reactants and their complexes was not altered by the polycation. Thus, emphasis in these experiments should be placed on the effects of PEI on glomerular permeability and enhancement of formation of GBM immune deposits, regardless of the mode of localization.

Alteration of glomerular charge and structure due to polycation binding might also influence the deposition of immune complexes during natural disease processes. For example, like PEI, positively charged proteins released by activated inflammatory cells and/or platelets, may bind to polyanions in the GBM and cause permeability defects that could affect the localization of antigens, antibodies, or their complexes in the capillary wall. We have shown by immunocytochemistry and ^{125}I-tagged protein binding that a human platelet secretory pro-

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tein, platelet factor four (which behaves like a polycation due to clustered lysine residues) binds avidly to the cell coats of glomerular endothelium and epithelium, and to the GBM (16). Whether or not this or other platelet secretory proteins can alter glomerular permeability remains to be determined.

It is of interest that Camussi et al. (17) have also demonstrated the localization of neutrophil cationic proteins in glomeruli affected by lupus erythematosus. Such glomeruli also showed a decrease of their affinity in vitro for colloidal iron, a cationic cytochemical probe for anionic sites. Because light microscopy was used exclusively to analyze the results by colloidal iron staining, it could not be determined which of the structures in the capillary wall was predominantly affected by cationic protein binding (17). Also, experimental models of immune complex disease have shown losses of polyanion in the GBM and increased glomerular permeability to anionic proteins before deposition of immune complexes (18) consistent with the postulated role for cationic proteins in glomerular immune complex deposition. Intensive study of the cationic proteins of leukocytes and platelets, the nature of their interactions with glomeruli and their pathogenic potential in immune complex deposition seems necessary.

Summary

It is known that polycations bind to and neutralize glomerular polyanions. Here we examine the effect of the polycation polyethyleneimine (PEI) on glomerular deposition of preformed immune complexes. Bovine serum albumin (BSA)–anti-BSA immune complexes made in 40 times antigen excess were administered following intravenous injection of PEI. Glomerular localization of immune deposits was assessed by quantitative immunofluorescence and electron microscopy and compared to controls receiving diluent without PEI followed by the same dose in immune complexes. In rats receiving PEI, deposits were localized within the glomerular basement membrane (GBM) of all peripheral capillary walls and in the mesangium. In controls, deposits localized exclusively within the mesangium in smaller amounts than after PEI. Thus, neutralization of glomerular polyanion by a circulating polycation enhances the deposition and alters the distribution of immune complexes in glomeruli.

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