

# Effect of Aminonucleoside Nephrosis on Immune Complex Localization in Autologous Immune Complex Nephropathy in Rats

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**ABSTRACT** The effect of increased capillary permeability on glomerular immune complex localization was studied in rats immunized with proximal tubular antigen (Fx1A) to induce autologous immune complex nephropathy (AICN). AICN rats were made proteinuric by injection or unilateral renal perfusion with aminonucleoside of puromycin (PA) before developing subepithelial complex deposits. Control AICN kidneys developed diffuse granular deposits of IgG and Fx1A on the subepithelial surface of the glomerular basement membrane (GBM) at 3 wk by immunofluorescence and electron microscopy, and deposits increased in subsequent weekly biopsies. In contrast, PA-nephrotic AICN kidneys developed few or no GBM deposits and a significant increase in mesangial localization of IgG and Fx1A during the period of PA-induced proteinuria. These alterations in complex localization were documented both in rats with PA nephrosis and in unilaterally PA-nephrotic kidneys compared with contralateral controls in the same animals, thus excluding any effect of PA on the immunopathogenetic mechanism in AICN as an explanation for these findings. The absence of GBM deposits closely correlated with reduced staining for polyanionic glomerular sialoprotein in proteinuric kidneys, since PA-perfused kidneys studied 2 wk after resolution of proteinuria demonstrated return of normal staining for sialoprotein

and development of subepithelial complex deposits similar to those in contralateral control kidneys. These studies demonstrate that properties of the glomerulus itself play an important role in determining the site of complex deposition in experimental AICN and suggest that electrophysical characteristics of the glomerular capillary wall may influence complex localization on the GBM.

## INTRODUCTION

Most immunologically mediated human renal diseases are believed to result from glomerular deposition of circulating immune complexes (1). The type and severity of the glomerular lesions produced are determined largely by the quantity and site of localization of immune reactants within the glomerulus (2). Studies in experimental acute and chronic serum sickness models in rabbits have indicated that several factors may influence immune complex localization, including complex size as determined by antigen:antibody ratio (1, 2), vasoactive amine release (2-4), and hydrodynamic (1, 3-5) and pharmacologic (6) factors. In membranous nephropathy, the most common cause of idiopathic nephrotic syndrome in adults (7), granular deposits containing IgG and having the ultrastructural characteristics of immune complexes are localized exclusively along the subepithelial surface of the glomerular basement membrane (GBM).<sup>1</sup> In selected

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<sup>1</sup> Abbreviations used in this paper: AICN, autologous immune complex nephropathy; Heymann nephritis; C3,  $\beta$ 1c- $\beta$ 1a, third component of complement; Fx1A, proximal tubular brush border antigen; GBM, glomerular basement membrane; IF, immunofluorescence microscopy; PA, aminonucleoside of puromycin.

patients, additional antigens have also been identified at this site and are believed to represent the antigenic component of immune deposits (8–12). Complexes in the subepithelial space are presumed to be derived from the circulation, although the mechanisms which regulate the localization of immune reactants at this site are not known.

Autologous immune complex nephropathy in rats (AICN, Heymann nephritis) is an established experimental model of membranous nephropathy in which the antigenic component of glomerular complex deposits is derived from proximal tubular brush border (13, 14). Recent studies in this model have shown that the earliest detectable epimembranous deposits are localized on the subepithelial aspect of the GBM adjacent to the basilar portion of epithelial cells and in the region of filtration slits beneath slit pore diaphragms (15, 16). This distribution of early epimembranous complex deposits corresponds to the localization of polyanionic glomerular sialoprotein on epithelial cells and in filtration slits (17–19). Recent physiologic and ultrastructural tracer studies have suggested a role for this negatively charged material in regulating the permeability of the glomerular capillary wall to circulating macromolecules in both normal and disease states (20–24). However, the effect of alterations in intrinsic properties of the glomerulus on the localization of immune complexes has not been previously investigated.

We have presented preliminary data indicating that AICN rats made proteinuric with aminonucleoside of puromycin (PA) before the earliest detectable complex deposition in the subepithelial space demonstrate a marked alteration in subsequent localization of immune deposits (25, 26). In the present report, our studies of complex localization in AICN rats with PA-induced proteinuria are described in detail. These observations indicate that the properties of the glomerulus itself which are altered by PA may be important determinants of glomerular complex localization.

## METHODS

**Induction of AICN.** ACIN was induced in 50–100-g male Lewis rats (Charles River Breeding Laboratories, Wilmington, Mass.) by a single injection in the rear footpads of 0.2 ml of an emulsion of equal parts of incomplete Freund's adjuvant containing 4 mg/ml of pulverized mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, Mich.) and 0.02 M phosphate-buffered saline, pH 7.3, containing 40 mg/ml of proximal tubular brush border antigen (Fx1A). Fx1A was isolated as described by Edgington et al. (27) from a homogenate of freshly prepared Sprague-Dawley rat renal cortices and lyophilized before use. Our previous studies of AICN induced by this protocol have demonstrated subepithelial glomerular deposits of IgG to be first detectable by direct immunofluorescence (IF) on day 21 and by electron microscopy on day 28 (16).

**Tissue processing.** Open renal biopsies were performed under ether anesthesia. Each cortical biopsy specimen was divided into three portions. Tissue for light microscopy was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin and periodic-acid Schiff stains. Histochemical staining for glomerular sialoprotein was carried out with colloidal iron at pH 1.8 (28) and Alcian blue at pH 1.6 (19), and was recorded as normal, reduced, or absent compared to controls. Tissue for IF was snap-frozen in isopentane in a dry ice-acetone bath, and 4-mm cryostat sections were prepared, stained, and examined as described elsewhere (29). Tissue for electron microscopy was fixed in glutaraldehyde, then postfixed in osmium for 60 min, followed by en bloc fixation in uranyl acetate for 30 min before embedding in Epon 812. Thin sections were studied on a Philips 300 electron microscope (N. V. Philips Gloeslampenfabrieken, Eindhoven, Netherlands). Results are based on analysis of over 700 sections and 225 electron micrographs taken of different portions of several glomeruli from representative animals in each group.

**IF procedures.** Direct IF was performed using procedures and controls previously described (29). Purified rat IgG (Miles Research Division, Miles Laboratories, Inc., Elkhart, Ind.), rat albumin (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) further purified by agarose column chromatography, and rat Fx1A prepared as described above were used in the preparation of rabbit antisera to these proteins. Antiserum to rat  $\beta 1c$ - $\beta 1a$  (C3) was prepared by a zymosan method (30). Rabbits immunized with Fx1A received three injections in the footpads and multiple sub- and intracutaneous sites of 10 mg of antigen in complete Freund's adjuvant (Difco Laboratories) given at weekly intervals and were bled 10 days after the last injection. Anti-Fx1A was heat inactivated (56°C, 30 min) and absorbed extensively with lyophilized whole rat plasma (2 mg/ml, 37°C for 1 h, 4°C overnight) until no precipitate developed and then with an equal volume of fresh pooled rat blood cells including erythrocytes, leukocytes, and platelets. Additional absorptions with purified rat IgG did not alter the IF staining characteristics of this antiserum.

The IgG fractions of all antisera were isolated from a 50% saturated ammonium sulfate precipitate by chromatography on DEAE-cellulose with a 0.02 M phosphate buffer, pH 7.5, concentrated by vacuum dialysis to 10 mg/ml of rabbit IgG as measured by radial immunodiffusion (31), conjugated with fluorescein isothiocyanate (BBL, Div. of Becton, Dickinson & Co., Cockeysville, Md.) by the dialysis method of Clark and Shepard (32), rechromatographed, and concentrated to 10 mg/ml of IgG in 10% pooled normal rabbit serum. Conjugated antibodies to rat IgG, C3, and albumin were monospecific by immunoelectrophoresis and micro-Ouchterlony double diffusion in 1% agarose; had fluorescein:antibody ratios of 0.140, 0.168 and 0.152, respectively (29); and were adjusted to precipitin titers of 1:4 before use. Anti-rat Fx1A was not reactive with rat plasma by double diffusion in gel, but at a dilution of 1:4 made two lines in 1% agarose against a 10 mg/ml suspension of Fx1A in saline (27, 33). By direct IF, this reagent reacted specifically with the luminal brush borders of proximal tubular cells in normal rat kidney and with glomerular deposits in partially eluted (see below) sections of AICN kidneys with 4+ glomerular IgG deposits. The titer of anti-Fx1A by direct IF on proximal tubular epithelium (1:64) was equivalent to the IF titer of antiserum to IgG determined on sections of AICN kidney with 4+ IgG deposits. Although glomerular staining for Fx1A was often detectable in uneluted sections of AICN kidneys biopsied 3 or more wk after immunization, staining was enhanced by partial elution of washed, unfixed, cryostat sections in 2.5 M potassium thiocyanate at 37°C for 2 h (13).

This procedure was employed routinely before staining for Fx1A. Specific staining with each antisera was blocked by prior incubation of sections with unconjugated antisera and by absorption with specific antigen.

Specific glomerular IF for IgG and Fx1A was recorded as 0–4+ for granular GBM deposits, with 4+ representing the maximal intensity of GBM deposits seen in proteinuric AICN rats at 10–14 wk (16). The intensity of mesangial staining was relatively uniform compared with the variation in GBM deposits. Mesangial staining was therefore graded by estimating the amount of mesangial areas occupied by granular deposits as follows: 0, no significant mesangial deposits compared to controls; 1+, 25%; 2+, 50%; and 3+, >50% of mesangial regions of most glomeruli containing deposits. All results were recorded by photomicroscopy using 60-s exposure times on high-speed Ektachrome (Eastman Kodak Co., Rochester, N. Y.) developed at ASA 400. Differences in IF findings between groups were analyzed by Student's *t* test (34).

IgG antibody titers to Fx1A in groups A, B, and C were determined weekly by indirect IF. Serial dilutions of serum were incubated for 60 min on normal rat kidney sections which were then washed and stained for rat IgG. Results were recorded as the highest tube dilution producing detectable tubular brush border staining. Differences between groups were analyzed by the Mann-Whitney test (34).

**Production of PA nephrosis and experimental design.** The characteristics of the experimental and control groups in this study are outlined for reference in Table I. The effects of increased glomerular permeability induced by systemic administration of PA on the localization of early subepithelial complex deposits in AICN were studied in 12 AICN rats given PA (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio) in normal saline, 100 mg/kg intravenously, on days 14 and 28 after Fx1A antigen injection (group A). The maximal individual dose of PA injected was 25 mg regardless of size. These rats had sustained proteinuria from days 21 through 35. Eight control AICN rats were injected with an equal volume of normal saline on days 14 and 28 (group B). A second group (group C) of eight age- and weight-matched Lewis rats immunized with complete Freund's adjuvant on day 0 received PA, 100 mg/kg intravenously, on the same days as rats in group A. Renal biopsies were performed on all rats in groups A, B, and C on days 21, 28, and 35 after immunization.

To exclude possible effects of PA administration on extrarenal factors potentially influencing complex localization, studies were also performed after exposure of only one kidney

to PA. Unilateral PA nephrosis was produced by selective perfusion of the left kidney with PA, 15 mg in 1.5 ml of normal saline, using techniques described in detail elsewhere (35, 36). Previous studies by one of us (Dr. Hoyer) have shown that proteinuria induced by this protocol develops 5–7 days after perfusion, lasts for 14–21 days, and originates exclusively from the perfused left kidney (35, 36). Left kidneys of 10 AICN rats were perfused with PA on day 14 after immunization with Fx1A, and bilateral biopsies were obtained on days 21, 28, and 49 (group D). The last biopsy was performed about 2 wk after PA-induced proteinuria had resolved. Biopsies from the perfused left kidneys are designated group D–L. Control biopsies from the nonproteinuric right kidneys of these animals are designated group D–R. Two additional control groups of rats were studied in a similar fashion after unilateral renal perfusion; left kidneys of four AICN rats were perfused on day 14 with saline alone (group E), and left kidneys of four age- and weight-matched Lewis rats immunized with adjuvant alone were perfused with PA on day 14 (group F).

**Other procedures.** 24-h urine collections were obtained in metabolic cages immediately before each biopsy in all animals, and urine protein excretion was measured by the sulfosalicylic acid method (37) using a whole serum standard. Urea and creatinine concentrations were determined by standard autoanalyzer techniques on serum samples obtained at the time of biopsy. The effect of PA on vasoactive amine activity was determined by measuring the bluing reaction induced in 30 min by intradermal injections of 1.0  $\mu$ g of histamine base or 0.5  $\mu$ g of serotonin in 0.1 ml of normal saline given after an intravenous injection of 1.0 ml of 0.5% Evans blue dye. Measurements were made 4 h and 1 and 5 days after administration of PA, 100 mg/kg intravenously, and 1 day after a second injection of PA given 7 days after the first. Control animals were injected with saline alone. Differences between groups were analyzed by Student's *t* test.

## RESULTS

**Urine protein and renal function.** All groups treated with PA had mean protein excretions exceeding 60 mg/day when biopsied on days 21, 28, and 35, and all animals in these groups were proteinuric. Mean urine protein excretions on days before each biopsy are shown in Figs. 1 and 2. Control AICN rats injected or

TABLE I  
Experimental Protocol for PA-Treated and Control Groups

Group	n	Day 0	Day 14	Day 21	Day 28	Day 35	Day 49
Rats given PA,* 100 mg/kg intravenously, or NS 10 ml/kg intravenously							
A	12	Fx1A-CFA	PA	Biopsy 1	Biopsy 2, PA	Biopsy 3	—
B	8	Fx1A-CFA	NS	Biopsy 1	Biopsy 2, NS	Biopsy 3	—
C	8	CFA	PA	Biopsy 1	Biopsy 2, PA	Biopsy 3	—
Rats with left kidney perfused with PA, 15 mg, or NS, 1.5 ml							
D–L	10	Fx1A-CFA	PA	Biopsy 1	Biopsy 2	—	Biopsy 3
D–R	10	Fx1A-CFA	—	Biopsy 1	Biopsy 2	—	Biopsy 3
E	4	Fx1A-CFA	NS	Biopsy 1	Biopsy 2	—	Biopsy 3
F	4	CFA	PA	Biopsy 1	Biopsy 2	—	Biopsy 3

\* PA = aminonucleoside of puromycin; NS = normal saline.

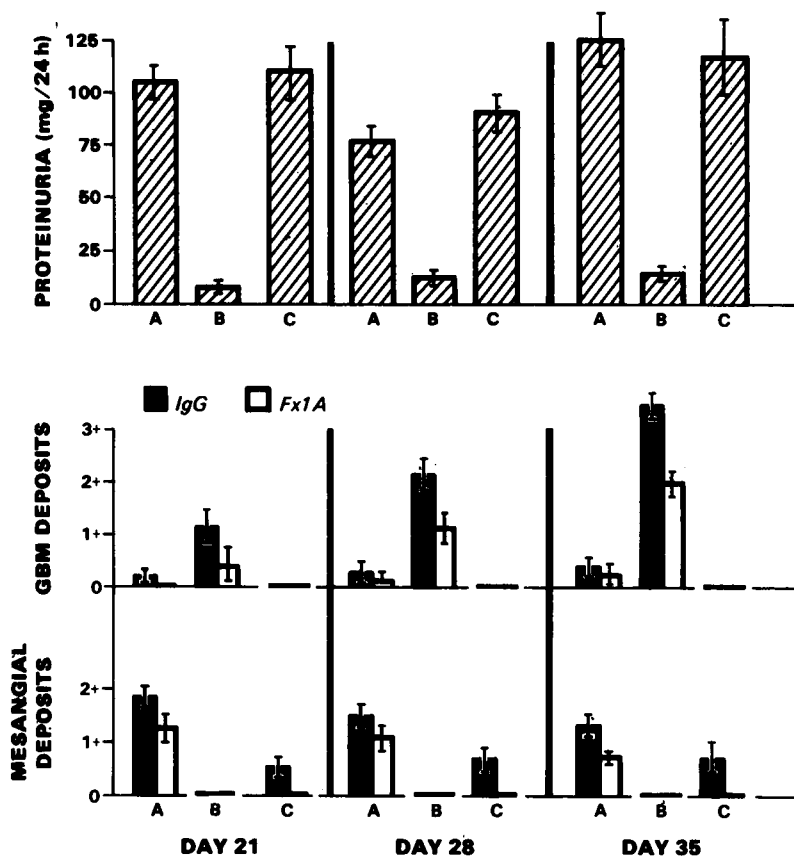


FIGURE 1 Urine protein excretion and mesangial and GBM deposits of IgG and Fx1A in groups A, B, and C on days 21, 28, and 35. Groups A and C received PA, 100 mg/kg intravenously, on days 14 and 28. All values are mean  $\pm$  SEM.

perfused with saline alone (groups B and E) had no significant increase in protein excretion during this period (Figs. 1, 2), a finding consistent with our previous results during the induction phase of AICN (16). Our previous studies have also indicated that increased proteinuria in unilaterally perfused rats is derived entirely from the perfused kidney (35), and that there is no significant increase in protein excretion due to AICN alone at 21 and 28 days (16). Therefore, proteinuria in rats in group D is depicted as coming only from PA kidneys (group D-L) at 21 and 28 days in Fig. 2 (recognizing that a very small percentage of the total protein excreted is derived from the right kidney). The mean urine protein excretions of perfused rats returned to nearly normal values by day 35 (group D:  $12 \pm 5$ , group F:  $9 \pm 6$  mg/day) and were not different from control values by day 49 (Fig. 2).

Serum creatinine and urea concentrations were not significantly different in rats receiving intravenous PA and controls at the time of biopsies on days 21 and 28 (Table II). By day 35, animals in groups A and C that had received PA had significantly reduced renal function

manifested by doubling of the serum creatinine concentrations and three-to-fourfold rise in urea nitrogen compared to nonproteinuric, saline-injected AICN controls in group B (Table II). Rats in groups D, E, and F perfused unilaterally with PA or saline had no significant increase in serum creatinine or urea (Table II).

*IF microscopy.* The patterns of complex localization in AICN rats given systemic PA and control groups were qualitatively similar to results in unilaterally PA-perfused animals and controls. These results of IF staining for IgG and Fx1A are shown in Figs. 1-4. Deposits of IgG and Fx1A in control AICN rats injected with saline (group B) and in nonperfused right kidneys of AICN rats (group D-R) were similar and corresponded closely with IF findings previously described during the early phase of AICN (16). Faint, finely granular deposits of IgG and Fx1A were present diffusely along the GBM of all glomeruli on day 21 and increased in biopsies on days 28 and 35 (Figs. 1-4). C3 was not demonstrable in GBM deposits on day 21 and was present in only trace amounts at days 28 and 35.

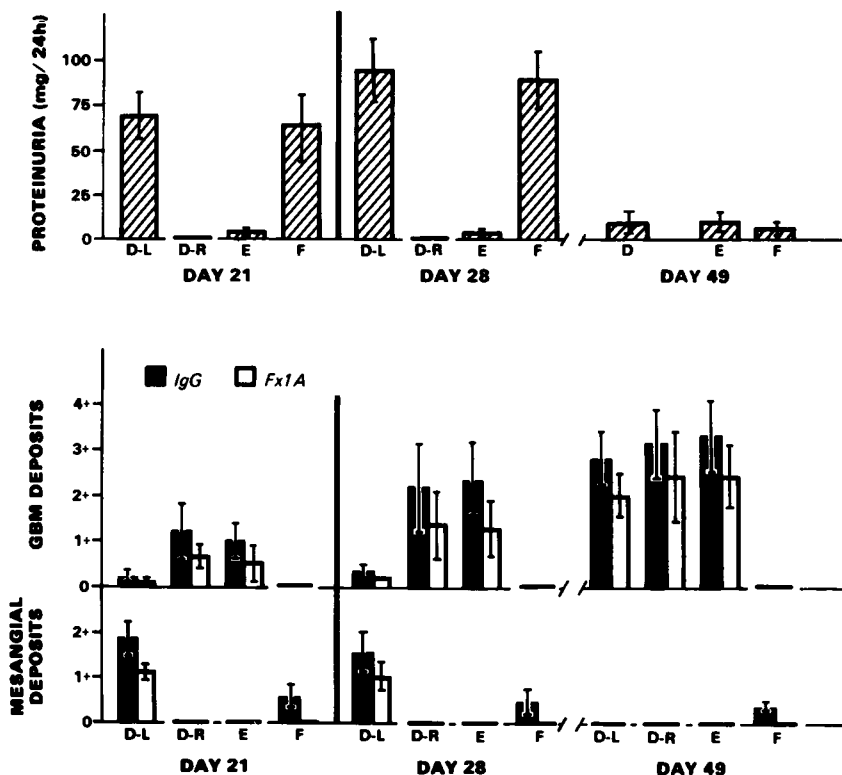


FIGURE 2 Urine protein excretion and immunofluorescence deposits of IgG and Fx1A on GBM and in mesangium in groups D, E, and F on days 21, 28, and 49. (Left) Kidneys in groups D-L and F were perfused unilaterally with PA on day 14, and group E was perfused with saline. All values are mean  $\pm$  SEM.

Mesangial deposits were not present in nonproteinuric AICN kidneys with IgG deposits on the GBM. The findings in saline-perfused kidneys in group E were not different from those in groups B and D-R, indicating that the procedure of unilateral perfusion had no effect on subsequent complex localization (Fig. 2).

The patterns of staining for IgG and Fx1A in proteinuric AICN kidneys in groups A and D-L were strikingly different from those of the control kidneys described above. The kidneys with proteinuria after systemic PA injection (group A) showed similar findings to kidneys selectively perfused with PA (group D-L). Glomeruli in AICN kidneys made proteinuric with PA before day 21 (groups A and D-L) had essentially no demonstrable deposits of IgG, Fx1A, or C3 on the GBM during the period of increased proteinuria (Figs. 1-4). In addition to the marked reduction in GBM deposits, proteinuric AICN kidneys in groups A and D-L also manifested a significant increase in deposition of both IgG and Fx1A in a granular pattern in the mesangium compared with nonproteinuric AICN controls in groups B, D-R, and E (Figs. 1-4). Mesangial staining for IgG was also significantly greater and more finely granular than that

seen in non-AICN control groups C and F that received PA ( $P < 0.05$  on days 21, 28, and 35) (Figs. 1-4). Mesangial staining seen in groups C and F with PA nephrosis alone was more confluent and nodular than that in AICN rats, as described previously by others (35, 38, 39). Mesangial staining for Fx1A paralleled that for IgG in proteinuric AICN kidneys and was in a similar pattern, although less intense (Figs. 1-4). No Fx1A staining was seen in glomeruli of control animals in groups C and F with PA nephrosis alone. No groups had significant staining for C3 in the mesangium. Proteinuric AICN kidneys in groups A and D-L frequently had staining for IgG on the luminal border of proximal tubular cells, presumably reflecting glomerular filtration of circulating anti-Fx1A antibody. IgG and albumin were present in rare tubular casts, and some granular and nodular staining for these proteins was present in mesangial and epithelial areas in groups A, C, D-L, and F as described by others in PA nephrosis (35, 38, 39).

These marked differences in complex localization between proteinuric and nonproteinuric kidneys described above were clearly apparent in each animal studied in group D when the proteinuric left kidney was compared with the nonproteinuric right kidney in

TABLE II  
Serum Creatinine, Urea, and Anti-Fx1A Levels at the Time of Each Biopsy

	Day 21	Day 28	Day 35	Day 49
Serum creatinine, mg/100 ml				
Group A	1.14±0.18 (12)*	0.96±0.15 (8)	2.38±0.42 (8)†	ND
Group B	0.78±0.16 (8)	1.00±0.17 (8)	0.91±0.11 (7)	ND
Group C	1.07±0.21 (8)	1.04±0.19 (6)	2.41±0.34 (6)†	ND
Group D	1.01±0.14 (10)	1.14±0.14 (10)	ND‡	1.06±0.18 (10)
Group E	0.94±0.17 (4)	0.98±0.21 (4)	ND	1.01±0.17 (4)
Group F	1.12±0.19 (4)	1.17±0.19 (4)	ND	1.18±0.21 (4)
Serum urea nitrogen mg/100 ml				
Group A	33.9±1.2 (12)	26.8±2.4 (8)	82.6±12.9 (8)†	ND
Group B	30.8±2.4 (18)	26.2±0.97 (8)	25.2±4.7 (7)	ND
Group C	31.9±1.8 (8)	29.1±1.9 (6)	76.1±10.8 (6)†	ND
Group D	34.1±1.4 (10)	28.9±2.6 (10)	ND	27.4±2.1 (10)
Group E	30.8±1.6 (4)	29.1±2.3 (4)	ND	28.4±2.6 (4)
Group F	36.2±2.9 (4)	32.8±3.1 (4)	ND	33.1±2.9 (4)
Anti-Fx1A antibody levels				
Group A	2.50±0.26 (12)	2.17±0.79 (8)	1.17±0.48 (8)†	ND
Group B	3.50±0.49 (8)	4.25±0.88 (8)	5.57±0.65 (7)	ND
Group C	0 (8)	0 (6)	0 (6)†	ND

Mean (±SEM) serum creatinine and urea concentrations in all groups, and anti-Fx1A antibody levels in groups A, B, and C, measured just before each biopsy. Groups A and C received PA systemically on days 14 and 28 and groups D and F were perfused unilaterally with PA on Day 14.

\* = N.

† = *P* value compared to group B <0.05.

‡ = Not done.

the same animal at days 21 and 28 (Fig. 4). In contrast, biopsies at day 49, 2 wk after resolution of proteinuria induced by PA perfusion, demonstrated 2–3+ GBM deposits of IgG and Fx1A in previously proteinuric kidneys in group D–L. GBM deposits were of similar intensity to those in the contralateral D–R and saline-perfused group E kidneys (Figs. 2, 4). Moreover, by day 49 the mesangial deposits seen in D–L kidneys during the period of increased proteinuria were no longer detectable (Figs. 2, 4). D–L kidneys differed from D–R and E kidneys on day 49 only by the presence of some residual tubular brush border staining for IgG (Fig. 4D).

**Electron microscopy.** Ultrastructural studies confirmed IF findings with respect to GBM deposits. As reported previously, no deposits could be seen in any group at 21 days (16). At 28 days, glomeruli in AICN control groups B, D–R, and E had numerous electron-dense deposits on the subepithelial aspect of the GBM, often in epithelial slit pore regions, with focal fusion of epithelial cell foot processes (Fig. 5A). In contrast, in PA-treated AICN kidneys in groups A and D–L there

was widespread fusion of foot processes (Fig. 5B). No deposits were found on the GBM on day 28 in group D–L (Fig. 5B) or on days 28 or 35 in group A animals. By day 49, extensive subepithelial complex deposition was apparent in groups D–R, D–L, and E. D–R and D–L glomeruli were not distinguishable by electron microscopy at day 49. Deposits were not seen in groups C and F with PA nephrosis alone. Mesangial deposits could not be clearly identified by electron microscopy in any group.

**Light microscopy.** Nonproteinuric control AICN kidneys in groups B, D–R, and E had no significant light microscopic changes at weeks 3, 4, and 5. Alcian blue and colloidal iron staining for glomerular polyanion was of normal intensity as previously reported (16). In contrast, all kidneys exposed to PA (groups A, C, D–L, and F) had markedly reduced or absent staining for polyanion at days 21, 28, and 35. By day 49, colloidal iron staining in previously proteinuric D–L kidneys had returned to normal intensity and was not perceptibly different from that in nonperfused D–R kidneys. No significant histologic abnormalities in the mesan-

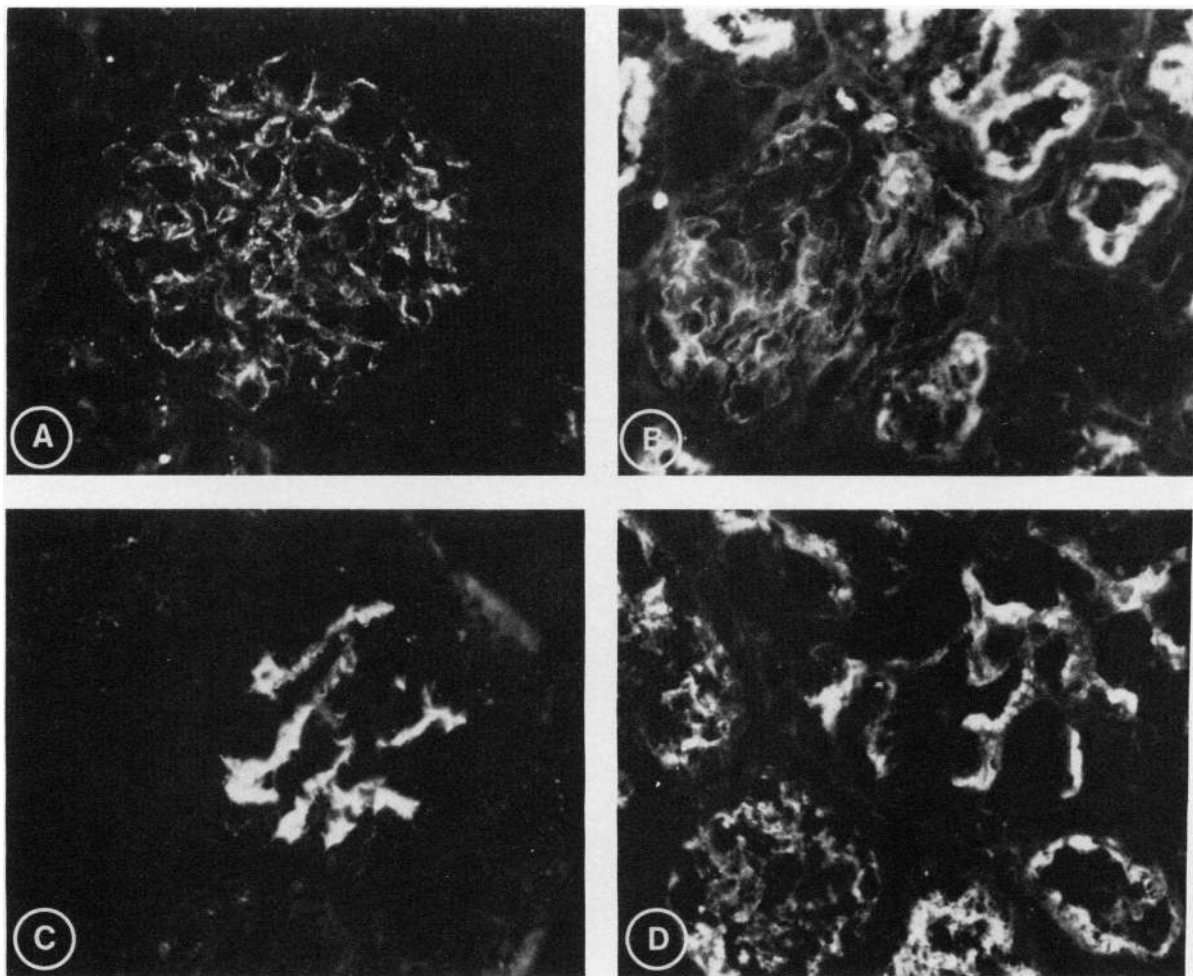


FIGURE 3 Immunofluorescent photomicrographs from biopsies on day 28. (A) Nonproteinuric AICN rat in group B with 2+ GBM deposits of IgG. (B) Same animal as A showing GBM deposits of Fx1A. (C) AICN rat in group A made proteinuric with PA and stained for IgG showing increased mesangial deposits. No GBM deposits are detectable. (D) Same animal as C showing staining for Fx1A in mesangium and absence of Fx1A on GBM. ( $\times 450$ )

gium were seen in AICN rats with mesangial deposits by IF compared with controls. PA nephrotic kidneys had only minor morphologic changes by light microscopy, including focal tubular dilatation, focal tubular casts, and periodic-acid Schiff-positive droplets in occasional glomerular and proximal tubular epithelial cells.

*Anti-Fx1A antibody levels.* Titers of circulating antibody to proximal tubular brush border determined by indirect IF in groups A, B, and C are expressed as tube dilutions in Table II. PA-treated AICN animals in group A had lower titers of antibody than nonproteinuric AICN controls in group B, but these differences did not reach statistical significance until day 35 (Table II). Despite the lower mean values in group A, there was considerable overlap, and several

animals without detectable GBM deposits in group A had higher antibody levels at days 21 and 28 than some animals in group B with 1–2+ GBM deposits. Animals in group C had no detectable circulating antibody by this technique (Table II).

*Vasoactive amine activity.* Measurements of the mean diameter of bluing induced by histamine and serotonin in PA-treated and normal rats at 4 h, 1 and 5 days, and 1 day after a second injection of PA 1 wk after the first demonstrated no effect of PA on increased capillary permeability induced by vasoactive amines either acutely or after PA-induced proteinuria.

## DISCUSSION

These studies demonstrate that kidneys of rats immunized with Fx1A fail to develop glomerular

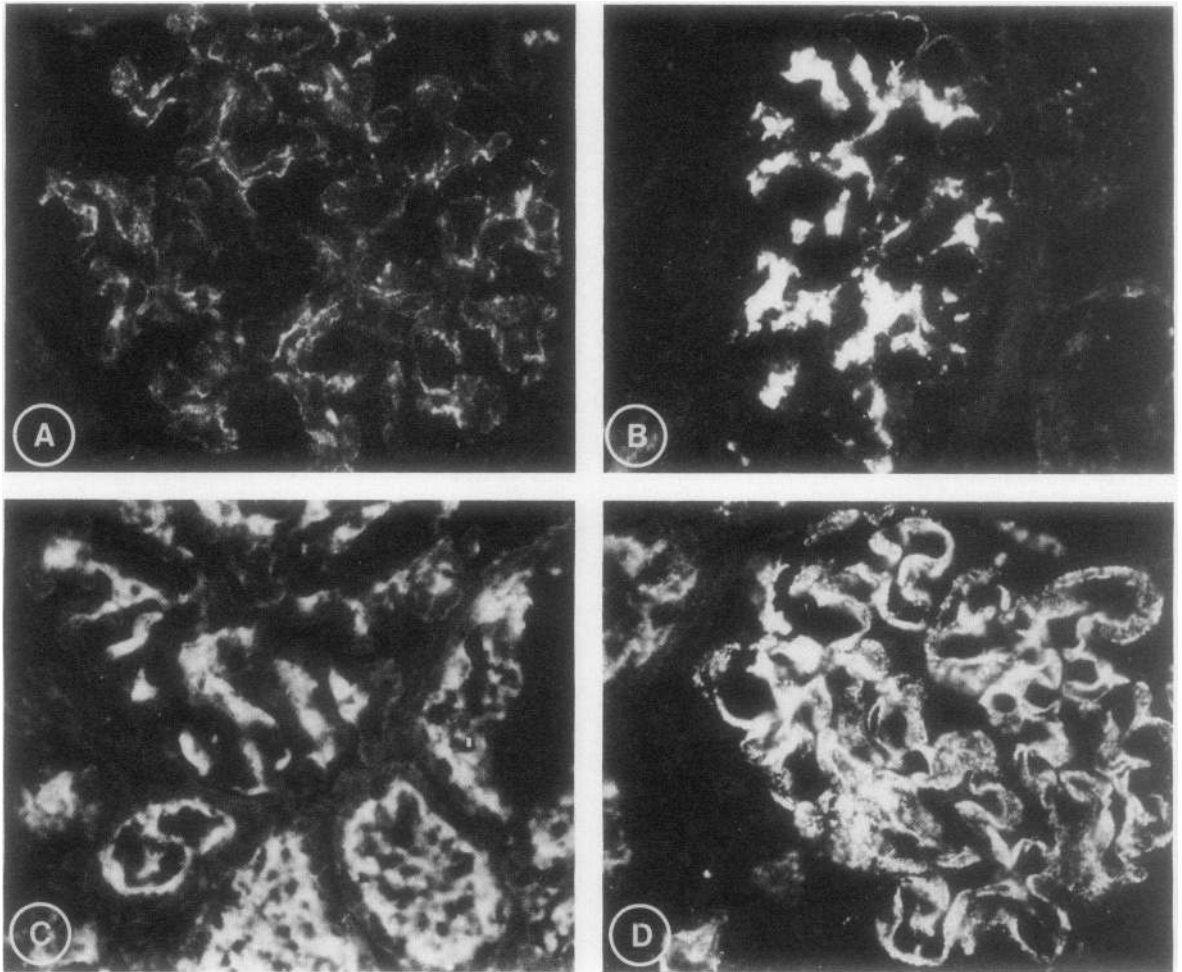


FIGURE 4 IF photomicrograph from AICN rat unilaterally perfused with PA. (A) IgG on GBM at day 28 in nonperfused R kidney in group D-R. (B) PA-perfused left kidney at day 28 from the same animal as A showing absence of GBM deposits and increased localization of IgG, predominantly in the mesangium. (C) Same biopsy as B, stained for Fx1A and showing localization of Fx1A in mesangium and no GBM staining. (D) Same kidney as in B at day 49, stained for IgG and showing development of typical membranous deposits after cessation of proteinuria. Mesangial deposits are no longer apparent. Some residual tubular brush border staining is present. ( $\times 450$ )

subepithelial complex deposits while proteinuric as a result of treatment with PA before the onset of glomerular complex deposition. After PA-induced proteinuria subsides, subepithelial deposits similar to those observed in control kidneys of AICN rats may be detected in these same kidneys. The studies in the unilateral model further demonstrate that this lack of epimembranous complex deposition in PA proteinuric kidneys occurs despite persistence of an ongoing immunopathogenetic process that results in epimembranous GBM deposits in nonproteinuric contralateral kidneys of these same animals. In addition, granular deposition of IgG and Fx1A were observed within the glomerular mesangium in AICN rats during the period of PA-induced proteinuria. These studies clearly

exclude an effect of PA on systemic factors that might influence the formation or composition of immune complexes. Hence, the changes in complex localization observed in PA-treated AICN rats demonstrate that properties of the glomerulus itself significantly affect the site and quantity of complex deposition. Such an effect has not been previously demonstrated.

The identification of antigens (9-12) and specific antibody to them (9, 40), in subepithelial GBM deposits in membranous nephropathy in man is consistent with the view that these deposits represent glomerular trapping of circulating, soluble immune complexes as apparently occurs in the acute and chronic serum sickness models in rabbits (1, 2, 41). Immune complexes have been directly visualized in the circulation, crossing the



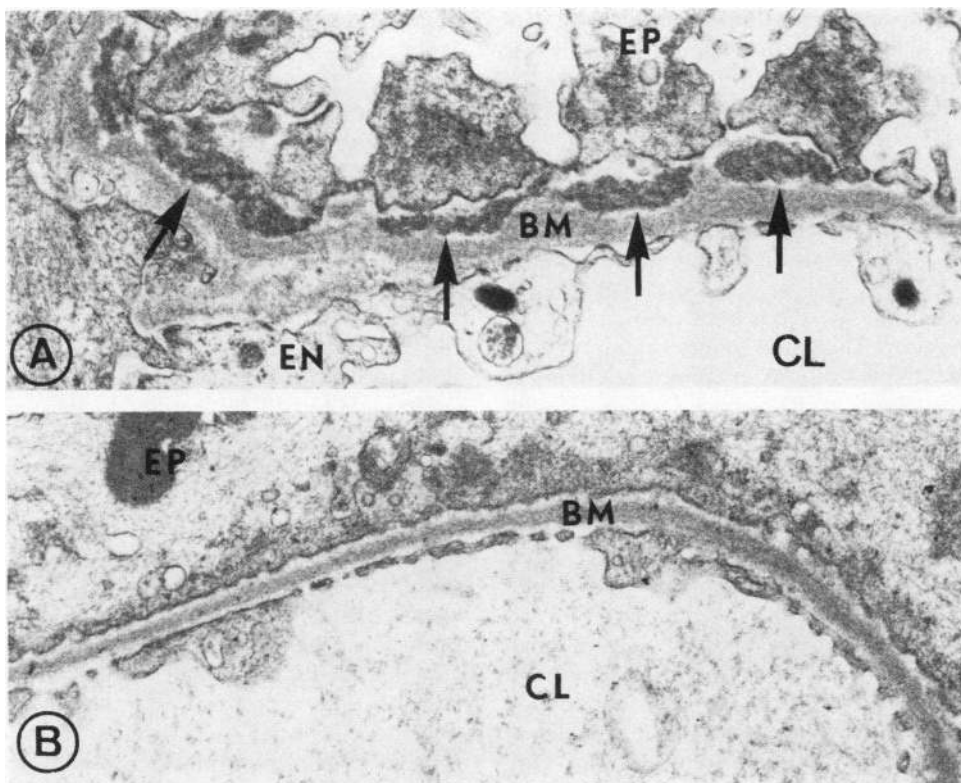


FIGURE 5 Electron micrographs from representative AICN rat in group D at day 28 comparing nonperfused right kidney (A) with PA-perfused proteinuric left kidney (B). Early complex deposits are present in the subepithelial space and in filtration slits in the right kidney (A, arrows). Deposits are absent in the proteinuric left kidney (B), which has extensive epithelial cell foot process fusion. BM, basement membrane; CL, capillary lumen; EP, epithelial cell; EN, endothelial cell; uranyl and lead.  $\times 19,700$ .

GBM, and localized in the subepithelial space in mice immunized with ferritin by Stilmant et al. (42). The view that subepithelial complex deposits in AICN also represent glomerular trapping of circulating immune complexes is supported by the presence of subepithelial granular deposits of tubular antigen and antibody after active (13, 34) or passive (33, 44) immunization against tubular antigens and by demonstration of tubular antigen (45) and antibody to it (14, 43, 45) in the circulation of AICN rats. However, recent studies by Van Damme et al. (46) have provided evidence that subepithelial immune deposits similar to those in AICN can be produced by direct perfusion of rat kidneys with antibody to Fx1A. These latter findings suggest strongly that the deposits seen in AICN may result from the reaction of circulating antibody with antigens within the glomerulus rather than deposition of circulating immune complexes.

Regardless of the precise mechanism by which subepithelial GBM deposits are formed in AICN, the basis for the marked reduction in epimembranous deposits in PA-treated rats in our studies has not been

established. Thus, several systemic variables which have been shown to influence the site and quantity of glomerular complex localization must be considered. These include changes in complex size and lattice formation due to alterations in the molecular weight of antigen or antibody or the antigen:antibody ratio (1, 2). In addition, alterations in clearance kinetics of circulating complexes due to changes in reticuloendothelial system function (47, 48), reduction and alkylation of the antibody component of circulating complexes (49), or administration of pharmacologic agents such as cortisone (6, 50) and drugs which affect vasoactive amine activity (1, 3, 4, 51) may alter complex localization. However, in the unilateral PA model, both kidneys were exposed to the same systemic milieu, and changes in complex localization were observed only in the PA-treated kidneys. These findings thus effectively exclude possible effects of PA on such systemic variables as the basis for the altered complex localization observed.

Several effects of PA on the structural and functional characteristics of the glomerulus warrant consideration

in interpreting our findings. Ultrastructural studies of glomeruli of rats made proteinuric with PA demonstrate extensive morphologic alterations in the epithelial surface of the capillary wall including separation of the epithelial cell layer from GBM (52, 53). However, such zones of epithelial detachment are focal and are present only in a minority of glomeruli in PA nephrosis (53). Hence they could not be responsible for the uniform absence of GBM deposits in PA-treated kidneys in our studies.

Hemodynamic changes have been shown to alter glomerular complex localization in other models (1, 5). A marked reduction in antibody or complex delivery to glomeruli consequent to PA-induced alterations in glomerular hemodynamics could decrease deposits in PA-treated kidneys. Bohrer et al. have characterized the hemodynamic changes induced by administration of PA in a dose comparable to that received by animals in our group A at the time of biopsy at 21 days (24). Their studies document a 40% reduction in glomerular filtration rate primarily due to a 60% reduction in the glomerular ultrafiltration coefficient ( $K_f$ ) and, to a much lesser extent, to a 20% reduction in glomerular plasma flow rate (24). It appears unlikely that a 20% reduction in glomerular plasma flow, and therefore delivery of antibody or complexes, could account for the very marked reduction in epimembranous deposits to essentially zero in PA-treated kidneys observed here. This conclusion also appears justified in light of other studies of the role of renal blood flow in glomerular complex deposition (54). Moreover, the presence of extensive antibody deposits on proximal tubular brush borders in kidneys of AICN rats with PA-induced proteinuria demonstrates that substantial glomerular delivery and filtration of antibody occurred without formation of GBM deposits. A possible contribution of PA-induced changes in  $K_f$  to our findings cannot be excluded with certainty, since the role of this property of the glomerular capillary wall in regulating localization of macromolecules is not known.

Recent studies have suggested a role for complement receptors demonstrated on the epithelial aspect of the GBM in man in contributing to complex localization at this site (55–57). However, glomerular complement receptors have not been demonstrable in the rat, and the presence of definite subepithelial complex deposits in AICN before localization of C3 (14, 16) further mitigates against a role for such receptors in this model.

An additional possibility is that the decrease in epimembranous complex localization observed was consequent to PA-induced alterations in electrophysical properties of the capillary wall. Histochemical and biochemical studies have shown a marked reduction in glomerular polyanion associated with the onset of proteinuria in PA nephrosis (19). The increased

fractional clearances of anionic dextran sulfates in PA nephrosis without increased clearances of neutral dextrans of the same sizes is also consistent with a loss of capillary wall charge in this model (24). Histochemical and ultrastructural studies demonstrate the major site of negative charge to be on the subepithelial aspect of the capillary wall in the polyanionic sialoprotein coating of epithelial cells and filtration slit diaphragms (17–19). Our present and earlier studies (16) and the studies of Schneeberger et al. (15, 58) demonstrate that the earliest detectable localization of complex deposits in AICN occurs at this site beneath epithelial cells and in filtration slits. Rennke et al. have shown that neutral and anionic ferritin molecules do not penetrate normal GBM, but that cationic ferritin molecules of the same size reach the subepithelial surface and accumulate as aggregates in filtration slits similar to the distribution of early deposits in AICN (21). Heparin-protamine polyelectrolyte complexes also localize in the subepithelial space and slit pores (59, 60). These studies suggest a role for capillary wall charge in the subepithelial localization of macromolecules in the glomerulus. Complex deposits in our studies were reduced or absent during the period of PA-induced reduction in polyanion staining and proteinuria and developed normally when proteinuria resolved and polyanion staining returned to normal. These observations suggest that glomerular capillary wall charge is also an important determinant of subepithelial complex localization in AICN.

The finding of granular deposits of IgG and Fx1A in the mesangium of PA kidneys in AICN rats is of interest. Schneeberger et al. have recently reported diminished mesangial uptake of colloidal carbon in AICN (61), a finding which may be related to the relative lack of mesangial immune deposits in rats with proteinuria due to AICN alone. Previous studies have demonstrated a marked increase in mesangial uptake of exogenous macromolecules from the circulation in glomeruli treated with PA or nephrotoxic serum (35, 36, 62). Although mesangial deposition of immunoglobulin has been noted in PA nephrosis by us and others (35, 38, 39), the mesangial deposits in PA-treated kidneys of AICN rats in this study exceeded those in PA-treated controls, were clearly granular as well as than nodular, and contained Fx1A, which was not found in the mesangium of controls. Since uptake of nonaggregated IgG is not increased in PA nephrosis (62), the mesangial IgG and Fx1A seen in AICN rats was presumably in a macromolecular, probably immune complex form. This finding suggests that circulating immune complexes containing tubular antigens are present in AICN. It further demonstrates that increased mesangial uptake of endogenous immune complexes in PA nephrosis appears similar to that previously shown with exoge-

nous macromolecules. As previously suggested (35, 36, 63, 64), this latter finding may be relevant to the pathogenesis of focal sclerotic mesangial lesions that develop in several chronic proteinuric disorders.

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