Histochemistry (1984) 81:243–246

Histochemistry

Preservation of fixed anionic sites in the GBM in the acute proteinuric phase of cationic antigen mediated in-situ immune complex glomerulonephritis in the rat

Y. Suzuki*, Y. Maruyama*, M. Arakawa*, and T. Oite**

* Second Department of Medicine and

** Department of Immunology of the Institute of Nephrology, Niigata University School of Medicine, Niigata 951, Japan

Received March 15, 1984 / Accepted May 26, 1984

Summary. Cationic antigens have been observed to bind with the negatively charged glomerular basement membrane (GBM). Using the cationic reagent polyethyleneimine (PEI), the distribution of glomerular anionic sites was evaluated ultrastructurally in the early stage (2 h–day 7) of cationic antigen mediated in-situ immune complex formation type glomerulonephritis (GN) in the rat. – Renal perfusion via the renal artery with 100 μg of cationized human IgG (pI > 9.5), followed by the i.v. injection of specific antibodies, led to an initial increase in urinary albumin excretion, subsequent massive globulinuria and the formation of numerous subepithelial deposits on day 7. – The most striking alteration in glomerular anionic sites was observed on the epithelial cell surface coat; the PEI deposition on the epithelial cell surface was almost identical to that in control glomeruli at 2 and 4 h after the induction of GN; thereafter, on day 7, a broad loss of anionic sites was observed on flattened epithelial foot processes. In contrast, fixed anionic sites of the laminae rarae of the GBM showed no apparent alterations in the distribution and number from 2 h to day 7 and did not disappear even in the lamina rara externa adjacent to subepithelial deposits. – These findings not only show that fixed anionic sites of the GBM, in contrast to the rapid decrease in those of the epithelial cell surface, are not completely neutralized or destroyed even in GN, in which cationic antigen participates in the in-situ formation of GBM-deposits. This also indicates that initial impairment of the charge-selective barrier of the GBM by the in-situ interaction between cationic antigen and antibody, is followed by the disfunction of the size-selective permselectivity of the GBM, ultimately causing massive proteinuria.

Introduction

The staining of the GBM with cationic dyes (Jones 1969; Michael et al. 1970; Kanwar and Farquhar 1979a, b, c; Schurer et al. 1978, 1980) and tracer studies using electron dense macromolecules such as cationized ferritin (Rennke et al. 1975; Kanwar and Farquhar 1979b; Farquhar 1980) have been applied to evaluate morphologically the function of the GBM as a charge-selective barrier for large circulating molecules. On the other hand, recent studies in experimental GN have shown that highly cationic proteins possess affinity for the negatively charged GBM and can serve as fixed antigens for circulating antibody to cause in-situ formation of immune complexes (Batsford et al. 1980a, b; Vogt et al. 1980, 1982; Oite et al. 1982, 1983). Such a mechanism has recently been noted also in human poststreptococcal GN (Vogt et al. 1983).

If circulating antibody reacts in-situ with cationic antigen at the site of anionic sites of the GBM, are anionic sites of the GBM thereby neutralized or destroyed? This study was designed to elucidate this issue ultrastructurally, using the combination of in vivo injection of and tissue immersion in highly cationic polyethyleneimine.

Materials and methods

A. In-situ immune complex formation type GN

Male Wistar rats aged 2 months were used. The left kidney of each animal was perfused via the renal artery with 0.5 ml of physiologic saline and successively with 100 μg of cationized human IgG (pI 9.5) [prepared according to the method by Danon et al. (1972)] in 0.2 ml of physiologic saline. Twenty minutes later, the rats were injected with 0.5 ml of rabbit anti-human IgG antiserum (Behringwerke, Germany) intravenously. The renal perfusion was performed at a flow rate of 0.5 ml/min (Oite et al. 1982, 1983).

Urinary protein excretion was determined by a turbidity method after precipitation with trichloreacetic acid in the urine from metabolic cages at 2, 4, 24 h and on day 4, 5, 7. Urine samples were qualitatively analyzed by immunoelectrophoresis using antiserum against rat serum proteins.

B. Histologic examination

Tissue processing for electronmicroscopic observation of glomerular anionic sites was performed as described previously with a slight modification (Suzuki et al. 1983). Animals were i.v. injected with 0.2 ml of 0.5% polyethyleneimine (PEI) (M.W. 40,000–60,000, Polyscience, Inc., PA) solution in distilled water, adjusted to pH 7.4 with HCl and to 400 mOsm with sucrose and sacrificed by bleeding from subclavian artery 15 min after the injection. The renal cortex was cut into small pieces, rinsed in 0.2 M cacodylate buffer, pH 7.4, 400 mOsm and then immersed in a 0.5% PEI solution (M.W. 1,800), pH 7.4, 400 mOsm for 30 min in an ice-water bath. Immersed tissue blocks were washed with the same cacodylate buffer and reimmersed in 2% phosphotungustic acid and 0.1% glutaraldehyde mixture, pH 7.4, 400 mOsm at room temperature for 60 min to obtain insoluble precipitates of PEI bound with glomerular anionic sites and simultaneous tissue fixation. After these procedures, cortical tissue blocks were washed with 0.2 M cacodylate buffer, pH 7.4, post-fixed with 1% osmic acid, dehydrated in series of alcohols and embedded in Epon. Epon-embedded tissue blocks were cut with glass knives in an ultramicrotome (MT-1, Sorvall Inc, USA) and unstained ultrathin sections were examined at 100 kV with a Hitachi H-600 electron microscopy.

Routine histologic examinations by light and electron microscopy were performed as described previously (Oite et al. 1983; Suzuki et al. 1983). For immunofluorescence, renal tissue was snap frozen in n-hexane cooled to −70° C in dry ice–acetone. Frozen

sections cut at 4 μm were rinsed in phosphate buffered saline, fixed in absolute acetone and stained with FITC-conjugated antibodies against rat IgG (Medical Biological Lab, Japan), C3 (Nordic Immunochemical, Holland) and human and rabbit IgG (Behringwerke AG, Germany). Stained sections were examined in an incident-type fluorescent photomicroscope (type BH-2, Olympus, Japan).

Results

1. Pathology in routine examinations

Light microscopy revealed no apparent abnormalities in glomeruli at 2 h but a segmental increase of endocapillary cells in some glomeruli at 4 h. Endocapillary proliferative GN accompanied by an infiltration of polymorphonuclear leucocytes appeared from day 1.

Immunofluorescence revealed a diffuse linear staining of human and rabbit IgG along the glomerular capillary wall at 2 and 4 h (Fig. 1 a). The fluorescence gradually increased in granularity and showed granular localization along the capillary wall on day 1. On day 7, discrete granular deposition of human, rabbit and rat IgG was observed diffusely along the glomerular capillary wall (Fig. 2 a).

By electron microscopy, electron dense deposits were observed from 12 h in the subepithelial spaces. These subepithelial deposits became larger and formed regularly distributed humps on day 7.

Significant proteinuria, which had been estimated as more than 30 mg/dl in preliminary studies, was observed from 4 h. On day 7, semiquantitative determination showed 300 mg/dl.

Immunoelectrophoresis revealed selective urinary excretion of albumin in concentrated urine samples before the renal perfusion of cationized human IgG. The increase in amount of albumin and a trace urinary excretion of IgG were observed in the urine at 2 h after the renal perfusion. On day 7, urinary excretion of almost all serum proteins was observed.

2. Alterations in glomerular anionic sites

At 2 and 4 h, the distribution of PEI bound anionic sites was essentially identical with those of glomeruli in control right kidneys and with those reported previously using a separate technique of injection or immersion of PEI; stained anionic sites were localized in the laminae rarae of the GBM, at the base and on the surface of the epithelial foot processes and scatteredly on the endothelial cell surface (Suzuki et al. 1983). The size of individual PEI aggregates in the laminae rarae seemed to be irregular, but there were no alterations in number as compared with control glomeruli (Fig. 1 b). As the loss of foot processes progressed, the epithelial cell surface was less stained with PEI. On day 7, a broad loss of anionic sites was observed on the flattened

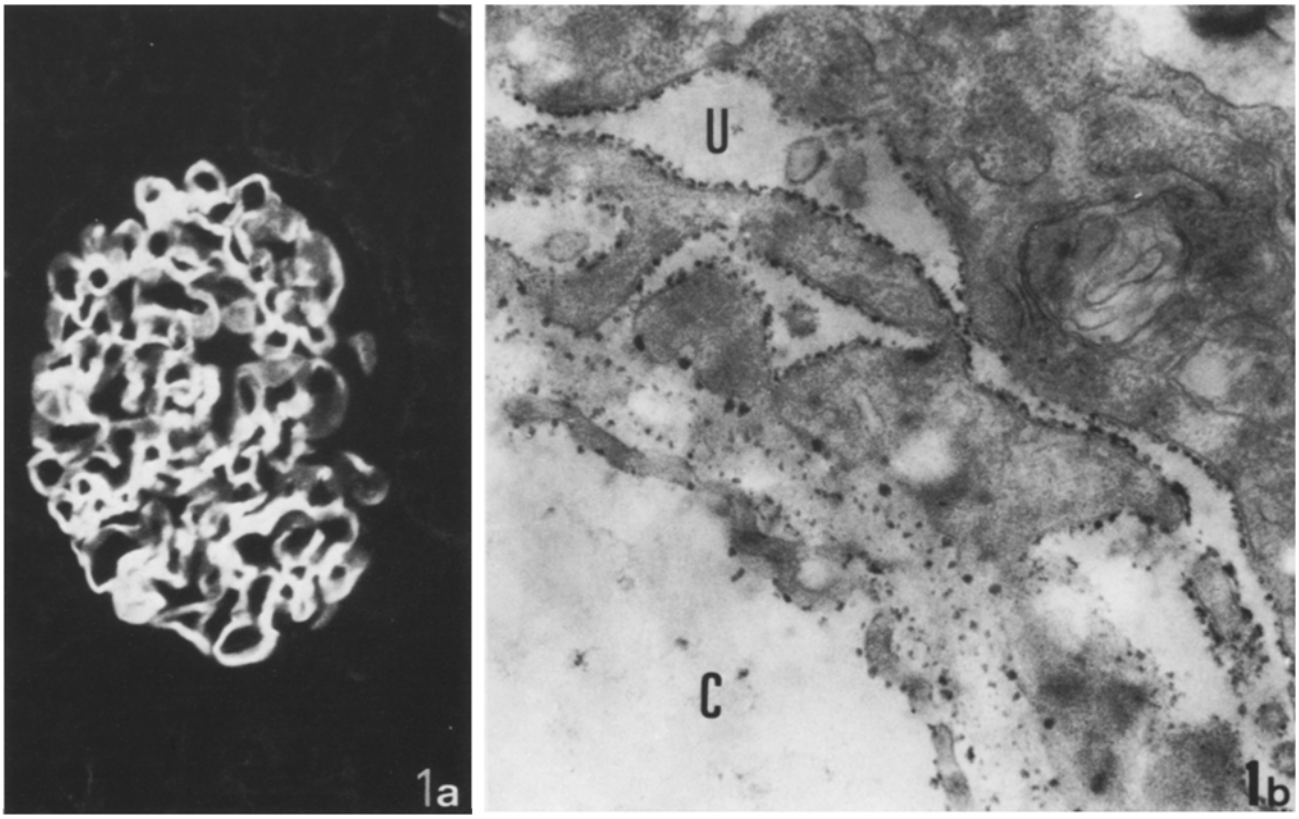

Fig. 1. a A cryostat section of kidney from an animal after renal perfusion with 100 μg of cationized human IgG and i.v. injected rabbit anti-human IgG antiserum 20 min later. At 2 h, a linear distribution of human IgG was seen along the capillary walls [stained with FITC-conjugated rabbit antibodies against human IgG] (×910). **b** A section of glomerulus from an experimental animal at 2 h showing fixed anionic sites stained with PEI; they are localized on the epithelial cell surface, at the base of foot processes and in the laminae rarae of the GBM, in the same distribution as in control glomeruli. Whereas immunofluorescence revealed in-situ antigen (cationized human IgG) – antibody (rabbit anti-human IgG antibodies) reaction (in **a**), electron dense deposits are not seen (×630,00). U-urinary spaces, C-capillary lumina

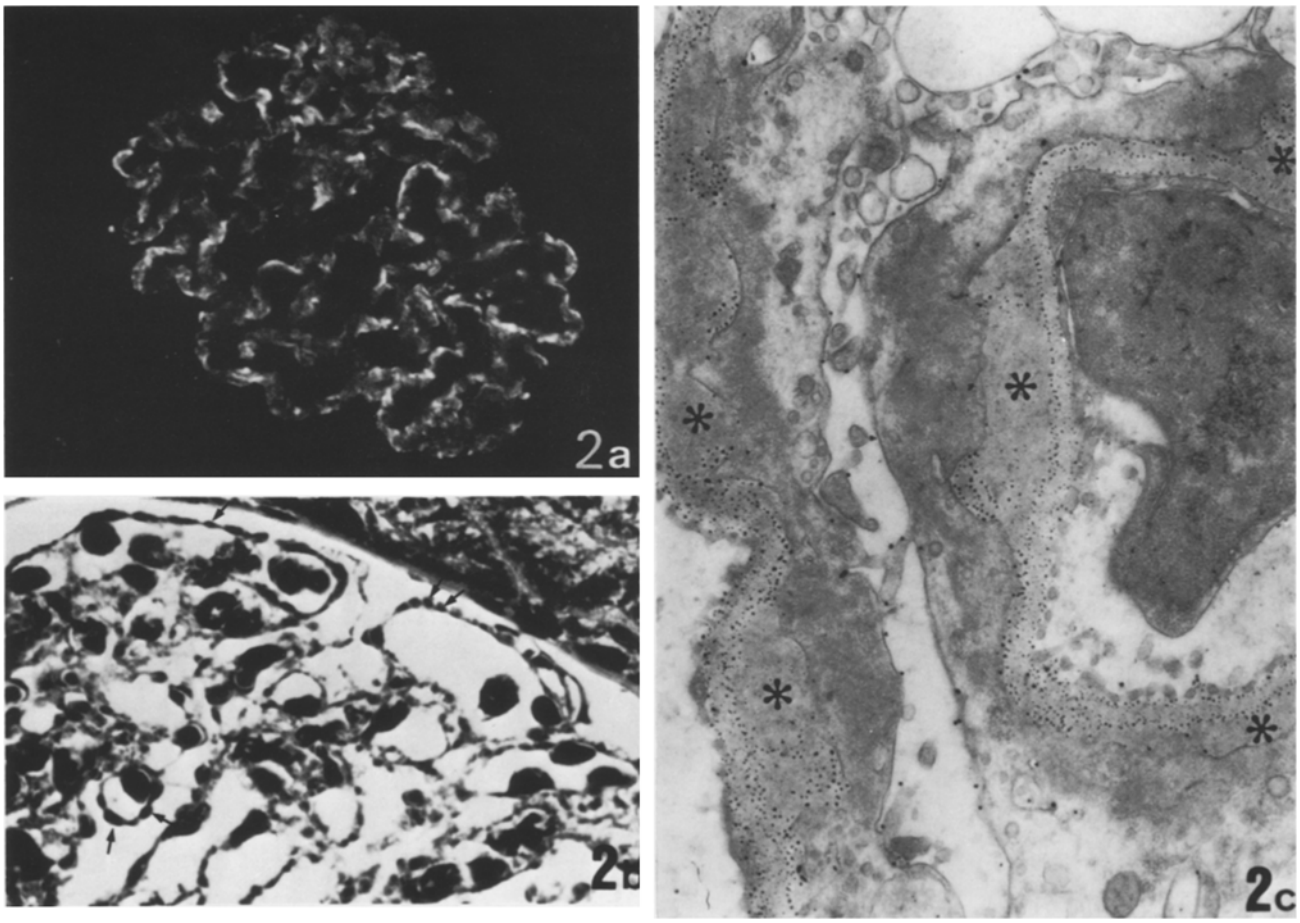

Fig. 2a, b. Kidney sections from an animal sacrificed on day 7, showing diffuse granular deposition of rabbit IgG along the glomerular capillary walls (**a** stained with FITC-anti-rabbit IgG antibodies, × 820) and endocapillary proliferative GN with a number of subepithelial deposits (arrows) (**b** periodic acid silver methenamine – Masson's trichrome stain, × 1,180). An ultrathin section of glomerulus with a number of subepithelial deposits (*), showing well stained anionic sites of the laminae rarae of the GBM and a complete loss of negative charges of the surface of flattened epithelial foot processes (× 21,600)

epithelial cell surface, except for a few PEI bound anionic sites at the base of foot processes. In contrast to the rapid decrease in anionic sites of the epithelial cell surface, those of the laminae rarae showed no essential alterations in distribution and number from 2 h to day 7 and did not disappear even in the lamina rara externa adjacent to subepithelial deposits on day 7 (Fig. 2c).

Discussion

It has been widely accepted that the perfusion of glomeruli with cationic substance leads to a decrease in glomerular anionic charge and the development of proteinuria and epithelial changes such as loss of foot processes (Seiler et al. 1975, 1977; Hunsicker and Shearer 1979; Hunsicker et al. 1981; Vehaskari et al. 1982). Moreover, recent studies in experimental GN have shown that highly cationic proteins bind with the negatively charged GBM and act as a target for circulating antibody (Batsford et al. 1980a, b; Vogt et al. 1980, 1982; Oite et al. 1982) to cause proliferative GN (Oite et al. 1983). However, there have been few morphological studies on the connection betwen cationic antigen-antibody immune complex formation and anionic sites of the GBM. In Heymann's GN in the rat, which is the prototype of human membranous nephropathy, we previously reported, using a PEI injection and immersion method, that subepithelial deposits closely interact with anionic sites of the lamina rara externa of the GBM, thereby contributing to the development and persistence of proteinuria (Suzuki et al. 1983). The combination of PEI injection and immersion, applied in the present study, is thought to be useful for the demonstration of all available glomerular anionic sites in a single specimen, since anionic sites of the laminae rarae and those of the epithelial cell surface are not simultaneously demonstrated by cationic macromolecules such as colloidal iron (Seno et al. 1983) or cationized ferritin.

Although the results obtained by immunofluorescence showed immediate antigen-antibody reactions after the administration of specific antibodies to the circulation, electron dense deposits were not observed at 2 and 4 h. Deposits with electron density appeared from 12 h at the subepithelial spaces and these subsequently became larger forming humps. These findings appear to indicate that immune complexes accumulate in the subepithelial spaces, from the anionic sites at which they are initially formed, and later develop into large aggregates.

An increased urinary excretion of albumin initially observed at 2 h might be due to the disturbance of the charge-selective permselectivity of the GBM, however, anionic sites

of the laminae rarae, as observed with PEI, were not completely masked or destroyed by the in-situ immune complex formation. This might be due to the mechanism of the rapid movement of immune complexes from the anionic sites where they are formed, to subepithelial spaces. Thus, in-situ reaction of antibody with cationic antigen at the anionic sites of the GBM may transiently influence their charge, but will not destroy their structure.

The loss of negative charge of the epithelial cell surface has been noted in aminonucleoside nephrosis (Michael et al. 1970; Caulfield and Farquhar 1978). Recently, a marked loss of anionic charge of the epithelial cell surface has been reported to precede the onset of the proteinuria of the nephrotic syndrome in rabbits given a single i.v. injection of cationized ferritins (Batsford et al. 1983). Thus, it is possible that the extensive loss of epithelial surface sialoglycoprotein might act as one of pathogenetic factors and contribute to persistent proteinuria in the present GN.

Massive globulinuria observed after the increase in urinary albumin excretion also strongly indicates the subsequent impairment of the size-selective barrier of the GBM in the present GN. It is likely that the matrix of the GBM determining the size-selective property alters in the three-dimensional structure by the formation and the development in size of immune complexes.

It was recently reported that polycations not only neutralize anionic sites, but also distort GBM gel structure, ultimately changing the porosity of the GBM (Barnes et al. 1983). Such a situation has been noted also by Hunsicker et al. (1983). But, such a mechansim seems hardly to participate in the present GN. Because, as Oite reported (1983), animals receiving only the renal perfusion of cationized human IgG showed no significant proteinuria.

Our results provide the important information that fixed anionic sites of the GBM are transiently influenced, in the net charge, but not completely masked or destroyed even in the cationic antigen-mediated in-situ immune complex formation type GN. Therefore, it should be noted, in prospective histochemical studies using cationic probes in human GN, the preservation of fixed anionic sites of the GBM does not exclude the participation of cationic antigens.

References

Barnes JL, Radnik RA, Gilchrist EP, Venkatachalam MA (1983) Size and charge selective permeability defects induced in glomerular basement membrane by a polycation [abstract]. Kidney Int 23:179

Batsford SR, Takamiya H, Vogt A (1980a) A model of in situ immune complex glomerulonephritis in the rat employing cationized ferritin. Clin Nephrol 14:211–216

Batsford SR, Oite T, Takamiya H, Vogt A (1980b) Anionic binding sites in the glomerular basement membrane: possible role in the pathogenesis of immune complex glomerulonephritis. Renal Physiol 3:336–340

Batsford SR, Sasaki M, Takamiya H, Vogt A (1983) Cationic macromolecule-induced nephrotic syndrome in rabbits. Lack of immune complex involvement. Lab Invest 49:260–269

Caulfield JP, Farquhar MG (1978) Loss of anionic sites from the glomerular basement membrane in aminonucleoside nephrosis. Lab Invest 39:505–512

Danon D, Goldstein Y, Marikovsky Y, Skutelsky E (1972) Use of cationized ferritin as a label of negative charges on cell surfaces. J Ultrastruct Res 38:500–510

Farquhar MG (1980) Role of the basement membrane in glomerular filtration: Results obtained with electron-dense tracers. In: Functional ultrastructure of the kidney. Academic Press, New York, p 31

Hunsicker LG, Shearer TP (1979) A model of acute reversible proteinuria induced by polycation infusion [abstract]. Kidney Int 16:783

Hunsicker LG, Shearer TP, Shaffer SJ (1981) Acute reversible proteinuria induced by infusion of the polycation hexadimethrine. Kidney Int 20:7–17

Hunsicker LG, Shaffer SJ, Bertolatus JA (1983) Hexadimethrine (HDM) induced proteinuria: lag between glomerular HDM binding and proteinuria [abstract]. Kidney Int 20:183

Jones DB (1969) Mucosubstances of the glomerulus. Lab Invest 21:119–125

Kanwar YS, Farquhar MG (1979a) Presence of heparan sulfate in the glomerular basement membrane. Proc Natl Acad Sci USA 76:1303–1307

Kanwar YS, Farquhar MG (1979b) Anionic sites in the glomerular basement membrane. In vivo and in vitro localization to the laminae rarae by cationic probes. J Cell Biol 81:137–153

Kanwar YS, Farquhar MG (1979c) Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membrane. Proc Natl Acad Sci USA 76:4493–4497

Michael AF, Blau E, Vienier RL (1970) Glomerular polyanion. Alteration in aminonucleoside nephrosis. Lab Invest 23:649–657

Oite T, Batsford SR, Mihatsch MJ, Takamiya H, Vogt A (1982) Quantitative studies of in situ immune complex glomerulonephritis in the rat by planted cationized antigen. J Exp Med 155:460–474

Oite T, Shimizu F, Kihara I, Batsford SR, Vogt A (1983) An active model of immune complex glomerulonephritis in the rat employing cationized antigen. Am J Pathol 112:185–194

Rennke HG, Cotran RS, Venkatachalam MA (1975) Role of molecular charges in glomerular permeability. Tracer studies with cationized ferritin. J Cell Biol 67:638–646

Schurer JW, Kalicharan D, Hoedemaeker PhJ, Molenaar I (1978) Demonstration of anionic sites in basement membrane and in collagen fibrils. J Histochem Cytochem 26:688–689

Schurer JW, Fleuren GJ, Hoedemaker PhJ, Molenaaer I (1980) A macromolecular model of the glomerular basement membrane. In: Functional ultrastructure of the kidney. Academic Press, New York, p 105

Seiler MW, Venkatachalam MA, Cotran RS (1975) Glomerular epithelium: structural alterations induced by polycations. Science 189:390–393

Seiler MW, Rennke HG, Venkatachalam MA, Cotran RS (1977) Pathogenesis of polycation-induced alterations ("fusion") of glomerular epithelium. Lab Invest 36:48–61

Seno S, Tsujii T, Ono T, Ukita S (1983) Cationic cacodylate iron colloid for the detection of anionic sites on cell surface and the histochemical stain of acid mucopolysaccharides. Histochemistry 78:27–31

Suzuki Y, Maesawa A, Matsui K, Oite T, Koda Y, Arakawa M (1983) Alteration of glomerular anionic sites by the development of subepithelial deposits in experimental glomerulonephritis in the rat. Virchows Arch [Cell Pathol] 44:209–222

Vehaskari VM, Root ER, Germuth FG Jr, Robson AM (1982) Glomerular charge and urinary protein excretion: effects of systemic and intrarenal polycation infusion in the rat [abstract]. Kidney Int 22:127

Vogt A, Schmidt U, Takamiya H, Batsford SR (1980) In situ immune complex nephritis and basic proteins, Proc EDTA. Pitman, London. Medical Publishing Co Ltd, Tumbridge Wells, Kent 17:613–620

Vogt A, Rohrbach R, Shimizu F, Takamiya H, Batsford S (1982) Interaction of cationized antigen with rat glomerular basement membrane: In situ immune complex formation. Kidney Int 22:27–35

Vogt A, Batsford S, Rodriguez-Iturbe B, Garcia R (1983) Cationic antigens in poststreptococcal glomerulonephritis. Clin Nephrol 20:271–279