Events That Stimulate Release of Thromboxane B_2 in Passive Heymann Nephritis (44084)

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Abstract. **The cause of proteinuria in passive Heymann nephritis has been attributed to the activation of the C_{5b-9} membrane attack complex following the antibody binding on the glomerular epithelial cell. Previous studies have shown an association between release of prostaglandin thromboxane B_2 (TxB_2) and proteinuria. Whether this release is dependent on antibody binding *per se,* or on secondary actions subsequent to antibody binding has not been clarified. The present study was designed to address this issue. Antibody binding event was experimentally separated from the proteinuria by employing a rabbit antibody which produces equivalent glomerular binding equal to that produced by a sheep antibody but without causing proteinuria. Comparisons were made with animals injected with the sheep antibody which produces all the hallmarks of the disease, including proteinuria. Animals injected with the rabbit antibody showed glomerular immunofluorescent deposits which were identical to the deposits produced by the control sheep antibody. However, rabbit antibody failed to produce the typical electron-dense subepithelial deposits, complement binding and proteinuria. Comparison of prostaglandin profile in isolated glomeruli revealed that TxB_2 was unchanged in rabbit antibody–injected glomeruli (compared with its nonimmune antibody control). On the other hand, glomeruli from sheep antibody–injected animals released 45% higher TxB_2 compared with their respective nonimmune antibody control. These data suggest that the binding of antibody *per se* may not be a sufficient stimulus for TxB_2 release. Subsequent events of subepithelial electron dense deposit formation, complement activation, and proteinuria are associated with TxB_2 release.** [P.S.E.B.M. 1997, Vol 214]

Passive Heymann nephritis (PHN) is a rat model of an antibody mediated non-infiltrative glomerular injury (1). Morphologically, it presents as granular immunofluorescent deposits (IF) along the basement membrane of the glomerulus and as electron dense deposits (EDD) in the lamina rara externa (LRE) of the basement membrane (2, 3). This model is equivalent to idiopathic membranous glomerulonephritis of humans. Following administration of heterologous experimental HN antibody (anti-Fx1A), the disease progresses through various stages which include antibody binding to the glomerular epithelial cell (within 1–2 hr), complement activation (day 3–5), formation of electron dense deposition in the basement membrane (day 12–14), and proteinuria (day 5–14) (4, 5).

The cause of proteinuria has been attributed to the direct injury of the plasma membrane of the glomerular epithelial cell by the antibody and the consequent C_{5b-9} membrane attack complex generated by complement activation (6–8). The role of other inflammatory mediators such as prostaglandins has also been examined. Release of TXB_2 has been shown to be associated with proteinuria (9–11). Whether it is the cause or a secondary effect of proteinuria has not been clarified.

Plasma membrane perturbances due to various causes result in activation of membrane phospholipase A2 which results in synthesis of prostaglandins (12–15). It is an open question whether antibody binding to plasma membrane *per se* is a sufficient stimulus to activate release of prostaglan-

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dins or whether a greater disturbance such as complement activation is required for this effect. The present study was designed to address this issue. Antibody binding and subsequent proteinuria events were experimentally separated by employing an antibody which produces equivalent glomerular binding but no proteinuria.

Materials and Methods

Induction of Passive HN. PHN was induced in male Sprague-Dawley rats (8 weeks old and 200 g) by a single intravenous injection of 0.5 ml of sheep anti-rat Fx1A or 0.5 ml of rabbit anti-rat Fx1A. Equivalent volume of respective nonimmune serum was injected in the control group. Sheep anti-Fx1A antibody was obtained from Dr. Bernice Noble, State University of New York at Buffalo (16). Rabbit anti-Fx1A was prepared as described previously (17). Its ability to induce deposits without proteinuria at the dosage used has been described in an earlier report (5). Prior to use, immune and nonimmune sera were heat-inactivated, at 56°C for 30 min and adjusted to 10 mg/ml of IgG. IgG concentration was determined by Biorad protein assay after removal of albumin by ammonium sulfate precipitation (50% saturation) on an aliquot of the anti-serum.

The titres of the sheep and rabbit anti-Fx1A antibodies were compared by radiobinding assay using [^{125}I]-labeled purified Heymann antigen gp600 (17) according to the protocol published previously (18). The results presented in Figure 1 show that the antigen binding titres of the two antibodies were comparable.

Animals were kept in metabolic cages 24 hr prior to being sacrificed. Urine was collected for protein and creatinine determination. During this period, food was withheld. Following the urine collection, blood was drawn in EDTA containing tubes. At 5 days post injection, a wedge shape portion of the kidney was collected and frozen for immunofluorescence study. A piece of kidney was fixed in 2% glutaraldehyde in phosphate-buffered saline (PBS) for electron microscopy evaluation. Urine protein was determined by Bio-Rad assay (Bio-Rad, Richmond, CA).

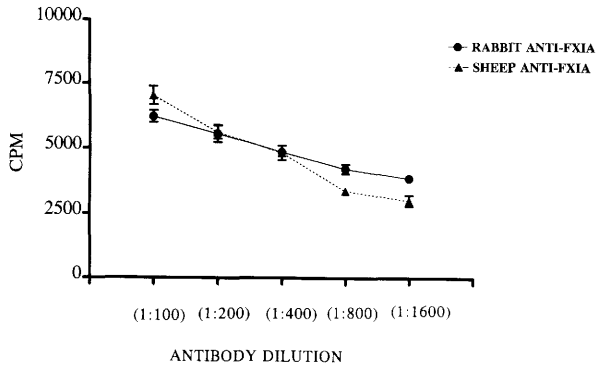

Figure 1. Comparison of antigen binding titres of rabbit anti-Fx1A and sheep anti-Fx1A antibodies against the purified antigen of Heymann nephritis, gp600. The detailed protocol has been described previously (18). Data are presented as means with limit bars showing standard errors. $n = 4$ at each dilution with respect to undiluted sera adjusted to 10 mg/ml IgG. The nonspecific background counts obtained in the assay were 422 ± 46, which was subtracted from each time point for presentation. The background counts were a mean of counts obtained in presence of 1:100 dilution of nonimmune rabbit IgG and sheep IgG.

Immunofluorescence Staining. Presence of rabbit IgG or sheep IgG in the kidneys were visualized by direct immunofluorescence as have been described previously (17). Sheep anti-rabbit IgG-FITC, rabbit anti-sheep IgG-FITC, and anti-C3-FITC conjugates were obtained from Cappel Laboratories (West Chester, PA). Cryostat sections (2–4 μm) of the kidney were collected on glass slides. Sections were washed with phosphate-buffered saline containing 130 m*M* NaCl, 10 m*M* PO_4, pH 7.4, 0.1% Tween-20 PBS-T. Antibody conjugates were spotted on the section and incubated for 30 min at room temperature in a humid chamber. The sections were washed twice with PBS-T for 10 min. Slides were mounted with 1:1 solution of glycerol and PBS and viewed under a fluorescent microscope with an epi-illuminator (Zeiss).

Electron Microscopy. Kidneys were fixed in 2% glutaraldehyde, 2% paraformaldehyde in cacodylate buffer, pH 7.4, and post-fixed with 1% aqueous osmium tetroxide. Following graded dehydration in ethanol, tissues were embedded in Epon for ultrasectioning. Thin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 1206 electron microscope.

Glomerular Eicosanoid and TxB_2 Synthesis. Kidneys were removed and placed in ice-cold RPMI medium (Sigma Chemical Co., St. Louis, MO). Renal cortices were removed and processed to isolate glomeruli as described previously using differential sieving (19). Glomeruli were incubated in 2 ml RPMI, at 37°C for 1 hr. Five micrograms of arachidonic acid was added to the incubation medium to provide adequate substrate. At the end of incubation glomeruli were separated by centrifugation at 2000*g*. Supernatant was removed to assay for prostaglandins and TxB_2. Glomeruli were dissolved by IN NaOH for protein determination using the Lowry method (20). Prostaglandins and TxB_2 were determined without prior extraction as described previously (14). Antibodies to PGE_2, 6-keto $PGF_{1\alpha}$, and TxB_2 were obtained from F. Pugliese, University of Rome, Italy (12, 13).

Results

Immunofluorescence microscopy obtained 5 days after injection of rabbit or sheep anti-rat Fx1A revealed discrete IgG deposits along the glomerular basement membrane (Fig. 2). The extent and distribution of the deposits were similar in rats which received sheep or rabbit anti-Fx1A. Presence of C3 was demonstrable in rats injected with sheep anti-Fx1A, but it was absent in rats injected with rabbit anti-Fx1A (Fig. 3). The kidneys of the control rat which received nonimmune sheep or rabbit serum did not show any deposits (data not shown).

In spite of similar glomerular immune deposits in the

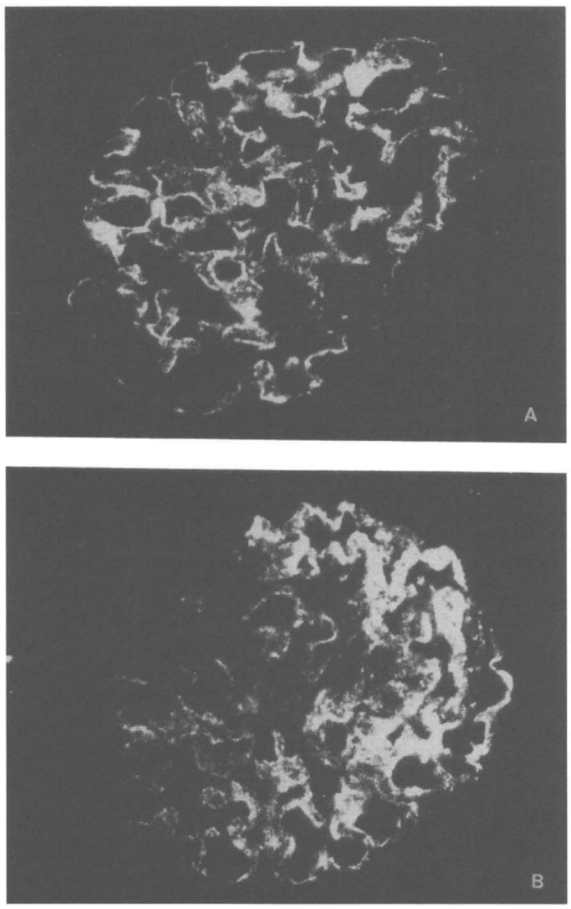

Figure 2. Immunofluorescent localization of rabbit IgG (A) and sheep IgG (B) in rat glomerulus 5 days after injection of rabbit anti-Fx1A and sheep anti-Fx1A antibody respectively. Discontinuous granular deposits of IgG were seen along the glomerular capillary loops in a similar pattern in Panels A and B. The staining after 5 hr of injection (not shown) was less intense, but more continuous.

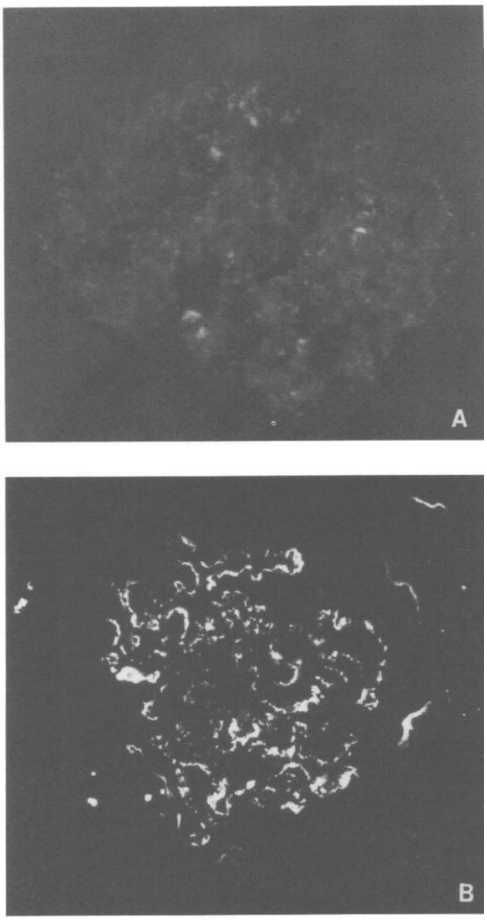

Figure 3. Localization of rat C3 by direct immunofluorescence in rat glomerulus 5 days after injection of rabbit anti-Fx1A (A) and sheep anti-Fx1A (B) antibody. The presence of C3 was essentially negative in animals injected with rabbit anti-Fx1A antibody (A). Note that the photomicrographs had to be overexposed to bring the glomerulus into view. On the other hand, rat C3 could be clearly demonstrated in animals injected with sheep anti-Fx1A (B).

capillary wall by immunofluorescence, we found different patterns of deposits by electron microscopy (Fig. 4). The animals injected with rabbit anti-Fx1A antibody did not show dense deposits. Subepithelial electron dense deposits were seen in all animals injected with sheep anti Fx1A. While animals injected with sheep anti-Fx1A were proteinuric, those injected with rabbit anti-Fx1A were nonproteinuric. There was a significant hyperlipidemia and hypoalbuminemia in the sheep anti-Fx1A injected rats (the analysis of hypoalbuminemia hyperlipidemic has been published separately) (21). A significant reduction in glomerular filtration rate, as measured by creatinine clearance, was observed (1.44 ± 0.2 ml/min in control group versus 2.33 ± 0.3 ml/min in proteinuric rats [$P < 0.001$]) (Table I).

Prostaglandins and TxB_2 profile of glomeruli isolated from rats 5 days following injection of two antibodies are presented in Figure 5. The glomerular PGE_2 and 6-keto $PGF_{1\alpha}$ were not different in either groups receiving sheep or rabbit anti-Fx1A compared with their respective control groups. However, there was a 45% increment in TxB_2 synthesis in the group that received sheep anti-Fx1A antibody compared with controls receiving sheep nonimmune IgG.

Discussion

In this study, two different polyclonal anti-Fx1A antibody raised respectively in the sheep and rabbit were used. Both antibodies induced the typical immunofluorescent IgG deposits along the glomerular basement membrane. However, only the sheep antibody was able to induce complement activation, electron dense deposits, and functional glomerular abnormalities such as significant proteinuria, production of TxB_2, and decrement in GFR. It was clear from this data that antibody binding alone was not a sufficient cause for the release of TxB_2 in PHN. The release of TxB_2 in the sheep antibody injected animals was co-associated with a decrease in Ccr, complement activation, presence of electron dense deposits, and proteinuria. It will be important to know which of these events triggers the release of TxB_2 and whether TxB_2 precedes or follows proteinuria.

TxB_2 has been considered previously to be a likely candidate to cause proteinuria in PHN (9–11). The correlation between TxB_2 synthesis and proteinuria has been reported in several experimental models. Zoja and Remuzzi

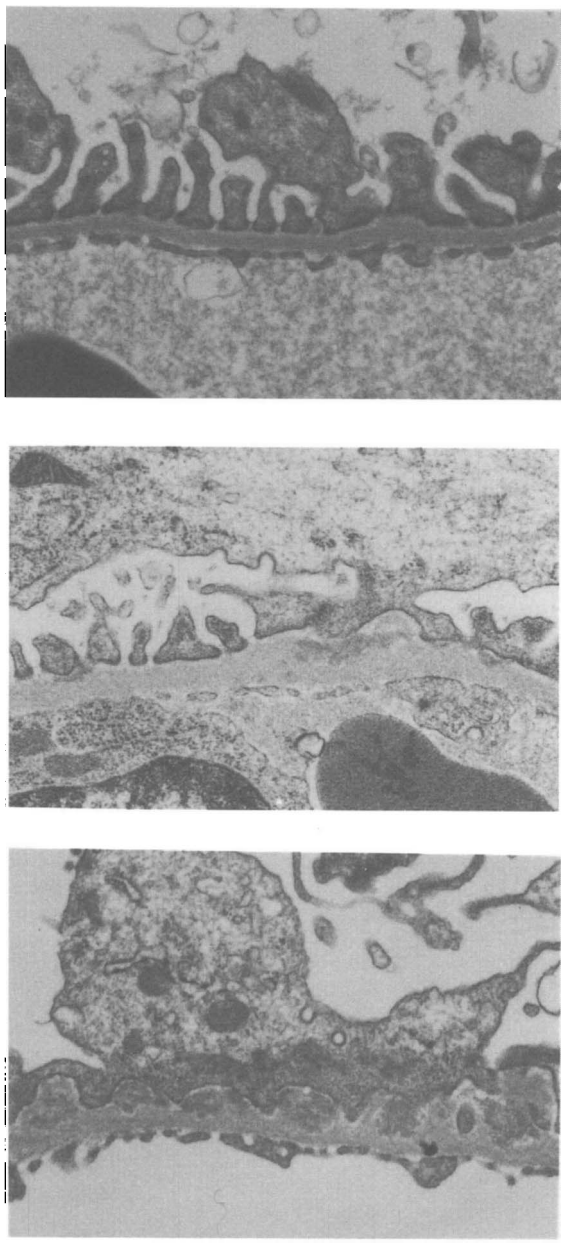

Figure 4. Ultrastructural (EM) visualization of electron-dense deposits in glomerular capillary wall of rats injected with rabbit anti-Fx1A (middle) or sheep anti-Fx1A antibody (bottom) 2 weeks after injection of antibodies. Uninjected control rat is shown in top panel. The capillary wall of rats injected with sheep anti-Fx1A antibody (bottom) show extensive dense deposits covering the entire length of the capillary wall. Also note the extensive foot process fusion. Rats injected with rabbit anti-Fx1A antibody showed only occasional deposits (middle) with essentially normal foot processes. Uninjected control rat showed the normal morphology (top).

(10) have found that the urinary PGE_2 and 6-keto PGF1α were not modified at the time the animals were proteinuric. However, urinary and glomerular TxB_2 synthesis increased significantly. Correlation between glomerular TxB_2 synthesis and proteinuria has been demonstrated in a model of aminonucleoside induced nephrosis. Remuzzi *et al.* (9) have demonstrated enhanced synthesis of glomerular TxB_2 synthesis and not 6-keto PGF1α or PGE_2 in this model. Lianos *et al.* (22) have reported a linear correlation between proteinuria and glomerular synthesis of TxB_2 in the experimental models of anti-GBM nephritis. In their study, glomerular PGE_2 and 6-keto PGF1α did not correlate with proteinuria. Thus, although it is accepted now that TxB_2 strongly correlates with proteinuria in rats, whether TxB_2 has a causal role in proteinuria is not defined. Zoja *et al.* (10) attempted to determine the causal role of TxB_2 synthesis in PHN by inhibiting TxB_2 by indomethacin and by a more selective Tx synthesis inhibitor UK 38,485. Although indomethacin significantly reduced proteinuria and urinary TxB_2, the specific inhibitor UK 38,485 failed to reduce proteinuria. This failure of the specific inhibitor could be due to its inability to reduce the urinary TxB_2 as was shown by these investigators. Our study supports the association of TxB_2 release with proteinuria. However, it sheds no light as to the role of TxB_2 in the causation of proteinuria.

The mechanism of TxB_2 release after the immune injury of the kidney can only be speculated at this stage. It is generally believed that TxB_2 is generated as a result of a series of enzymatic reaction initiated by the hydrolysis of membrane phospholipid into the precursor arachidonic acid. The hydrolysis is triggered by membrane perturbations as has been shown to occur by polycation binding to the membrane polyanions of the mesangial cells (13, 14) and epithelial cells of the glomerulus (12). Similar perturbation could be expected from the *in situ* antibody binding to membrane antigens as is the case in PHN. However, our results with the rabbit antibody indicated that there may be a threshold to this trigger. A high cross-linking and therefore deformation of the membrane antigens may be required for the stimulation of phospholipid (Lecithin) hydrolysis. The antibody raised in sheep, which is more separated from the rat in the phylogeny than the rabbit, would contain greater heterologous specificities to rat kidney antigens than the rabbit antibody would. This may explain the greater potency of the sheep antibody compared with the rabbit antibody. The greater cross-linking of membrane antigen has been shown to be critical in activating the complement system in PHN (5, 23), which subsequently results in tissue injury and consequent proteinuria (7, 8). Thus, in the PHN model the release of TxB_2 may be an event dependent on the antigen cross-linking of the cell membrane antigens, but independent of complement activation and proteinuria. This contention is supported by experimental observations of others (10) of an early release of TxB_2 after injection of sheep antibody which correlates with a transient proteinuria in the absence of complement activation. However, this remains to be confirmed.

Very little information is available about the source of TxB_2 in the PHN disease model. The immune reaction of PHN is limited to the glomerular epithelial cell, with minimal cell infiltration from the blood. We have previously shown (12) that the glomerular epithelial cells in culture produce a host of prostaglandins, and some of these are inducible by nonimmunologic insults. Cybulsky *et al.* (15) have shown activation of phospholipase in the cultured glo-

Table I. Comparisons of Renal Function in Rats Injected with Sheep Anti-Fx1A and Rabbit Anti-Fx1A Antibodies after 5 Days of Injection

	Dose (mg)	Rat wt (g)	Ccr (ml/min)	Urinary Protein (mg/24 hr)
Rabbit anti-rat Fx1A IgG (n = 6)	5	237 ± 8.0	1.53 ± 0.16	25.6 ± 6.3
Rabbit non-immune IgG (n = 6)	5	242 ± 2.6	1.96 ± 0.4	28.12 ± 4.3
Sheep anti-rat Fx1A IgG (n = 10)	5	242 ± 8.2	1.44 ± 0.2[a]	555 ± 31.5[b]
Sheep non-immune IgG (n = 10)	5	242 ± 3.0	2.23 ± 0.3	16.8 ± 1.7

Note. Data are presented as mean ± SEM.
[a] $P < 0.1$; [b] $P < 0.001$, compared with the respective nonimmune IgG controls.

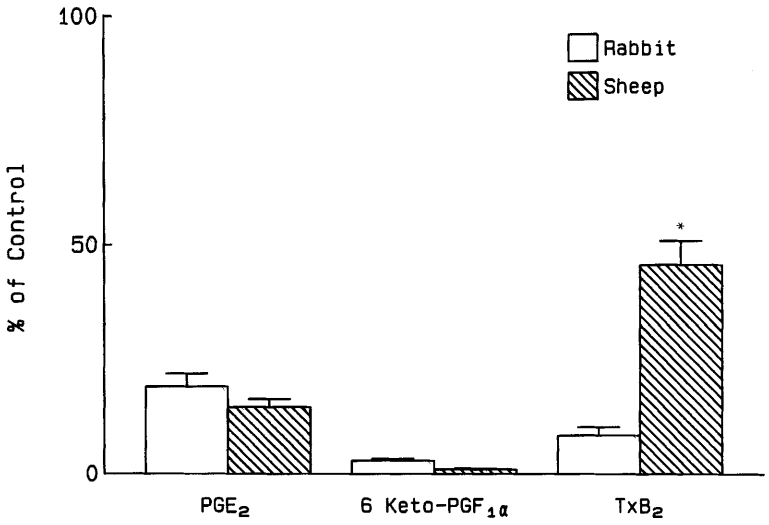

Figure 5. Prostaglandin (PGE_2, 6-keto $PGF_{1\alpha}$) and thromboxane (TXB_2) profile of glomeruli from rats 5 days after injection with rabbit and sheep anti-Fx1A antibodies. Data are presented as mean with bars showing standard errors. $n \geq 5$ in each group. All the eicosanoids tested increased to above control levels by both the antibodies. There was no significant difference in PGE_2 and 6-keto $PGF_{1\alpha}$ values between the animals injected with rabbit anti-Fx1A and sheep anti-Fx1A. However, there was a statistically significant difference ($P < 0.01$) in the TXB_2 levels between the two groups.

merular epithelial cell when incubated with the membrane attack complex, the final event in PHN which causes cytopathic effects in this cell. Therefore, it is likely that this cell may be the source of TxB_2. However, recently it has been observed that glomerular cells under stress elicit potent cytokines such as TGFβ (24). In light of this knowledge, it is possible that other cells of the glomeruli could produce TxB_2 due to the paracrine activation by the glomerular epithelial cells. In a related study by Katoh *et al.* (25) in the PHN model, it was found that the inflammatory mediator leukotrine LTD_4 was increased and the source of this mediator were the Ia(+) mesangial cells. Since arachidonic acid is the common precursor for leukotrienes and prostaglandins, it is a possibility that the Ia(+) cells in the mesangium may be the origin of TxB_2 also.

In summary, our experiments show that antibody load on cell membranes may not be the sufficient cause for TxB_2 release from glomeruli. There needs to be a qualitative difference in the antibody that can cause release of TxB_2. The qualitative differences seem to reside in the "heterologousness" of the antibody. The same quality in the antibody would also impart the ability to activate complement, to produce electron dense deposits, and to cause proteinuria. Whether the TxB_2 is the cause of proteinuria or is the effect of tissue damage, which also leads to proteinuria, still remains an open question and will be addressed in future studies.

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