

Glomerular injury induced in mice by intraperitoneal injection of Shiga-like toxins

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Background & objectives : Shiga-like toxins I and II (Stx1 and Stx2) play an important role in the pathogenesis of renal disease by causing renal microvascular injury. A murine model was used to study glomerular lesions produced by Stx1 and Stx2.

Methods : Swiss albino mice of the Rockefeller strain were inoculated intraperitoneally with LD₅₀ doses of endotoxin-free Stx1 or Stx2 and observed for signs of disease. Samples of renal cortical tissue from mice were examined with the electron microscope.

Results : The mice developed systemic and neurological symptoms including hind limb paralysis and generalised convulsions. Renal arteriolar damage and glomerular endothelial cytoplasmic swelling, vacuolation, lysis and intravascular coagulation were present and resembled the microangiopathy seen in renal biopsies from patients.

Interpretation & conclusions : These experiments establish the role of Stx1 and Stx2 in glomerular vascular injury and provide a model for studying the pathogenesis of Shiga-like toxin related microangiopathy.

Key words Capillary - endothelium - microvasculature - renal - Stx1 - Stx2

Haemolytic uraemic syndrome (HUS) is a well known complication of infection by *Shigella dysenteriae* serotype I and certain strains of *Escherichia coli*¹⁻³. The syndrome is due to glomerular damage⁴⁻⁶ by bacterial endotoxin⁷ and exotoxins including Shiga toxins : Stx, Stx1 and Stx2⁸⁻¹⁰. Previous experimental models have shown thickening of the glomerular capillary wall, congestion of the glomerular capillaries, swelling and detachment of the glomerular capillary endothelial cells and formation of intravascular fibrin in response to lipopolysaccharide (LPS)⁷, Stx1¹¹ and Stx2 toxin

producing *Esch. coli* 0157:H7^{10,12}. The effect of Shiga-like toxin alone on mice has not been studied and ultrastructural changes in glomerular capillary endothelium in response to Shiga-like toxins have not been described in detail. The present study was designed to identify the effects if any, of endotoxin-free Shiga-like toxins (Stx1 and Stx2) on glomerular capillaries of Swiss albino mice.

Material & Methods

Shiga-like toxins, Stx1 and Stx2 had been purified¹³ from *Esch. coli* strain C600-933J and C600-933W,

derived from apathogenic *Esch. coli* C600, lysogenised with bacteriophages 933J and 933W, which encode Stx1 and Stx2 respectively. Samples of toxin in all dilutions used had been checked and found to be free from LPS contamination by the *Limulus amoebocyte lysate* test¹⁴. The 50 per cent lethality dose of Stx1 and Stx2 for 4-6 wk old Swiss albino mice of the Rockefeller strain had been determined by the method of Reed and Muench¹⁵. The 50 per cent lethal dose for Stx1 was 150 ng and for Stx2 4ng. The difference in these two doses was as expected¹⁶.

Experimental protocol : Two groups of 15 mice each were inoculated intraperitoneally with LD₅₀ dose of Stx1 and Stx2. Six mice were inoculated with an equivalent volume of sterile phosphate buffered saline (PBS) at pH 7.2 as controls. The mice were observed for ruffled fur, tachypnoea, fever, anorexia, muscle weakness, paralysis, diarrhoea, anuria and haematuria, thrice daily till the onset of illness and continuously thereafter till death or complete recovery. All surviving mice were observed thrice daily for 2 wk. The Ethics Committee of the Institute approved the study.

Electron microscopy : In each test group, renal cortical tissue was obtained from 6 mice, 4 of which were sacrificed 1-3 h after the onset of illness and 2 immediately after death following the illness. Renal cortical tissue was also obtained from 4 PBS treated controls. Mice were sacrificed by an overdose of ether anesthesia. Tissue was fixed in 2.5 per cent gluteraldehyde, postfixed in osmium tetroxide and embedded in araldite. Semithin sections were screened under the light microscope and two blocks from abnormal areas of each animal were selected for electron microscopy. Ultrathin sections were cut on an LKB UM4 ultramicrotome (Bromma, Sweden) with a diamond knife (Diatome, Switzerland), stained with uranyl acetate and lead citrate and examined with a Philips EM201C electron microscope (Eindhoven, The Netherlands). Sections of the glomerular capillaries were evaluated for vascular congestion and morphological evidence of endothelial cell damage such as endothelial swelling, organelle damage, rarefaction, fragmentation or vacuolation of cytoplasm. The presence of platelets and neutrophils

in the lumen was also looked for. In all, 189 glomerular capillary cross sections were examined including 67 from controls, 65 from Stx1-treated mice and 57 from Stx2-treated mice and the number with lesions was noted. Chi-square test was done to compare the frequency of vascular damage between the groups. Bon-Ferroni correction was incorporated in *P* values. Lesions present in the peritubular capillaries, arterioles and renal tubules were recorded.

Results

Nine of the 15 mice inoculated with Stx1 developed signs of systemic disease including lethargy or restlessness, anorexia, ruffled fur, shivering, shallow and rapid respiration, 48-92 h post injection. Within the next 2-3 h, 7 of these mice developed neurological signs of hind-limb weakness, rigidity or paralysis and jerky rhythmic movements of the limbs. Three mice had severe, generalised convulsions that proved fatal. In non-fatal cases recovery was complete and occurred within a further 3 h. There were no gastrointestinal or renal symptoms such as diarrhoea, anuria, oliguria or haematuria during the entire period of observation. Eleven of the 15 mice inoculated with Stx2 developed an illness similar to that caused by Stx1. Nine of the affected mice developed fatal neurological complications. In neither test group did death occur in mice developing systemic illness alone.

Gross & light microscopy : The kidneys of mice injected with Stx and those of controls were grossly normal at necropsy. One micron sections showed endothelial swelling and congestion in few glomeruli of toxin-treated mice. There was patchy vacuolar change of the proximal tubules in mice injected with toxins, but not in the controls.

Ultrastructural changes in the kidney : Glomerular capillaries of control mice were generally normal and lined by flattened fenestrated endothelial cytoplasm with only mild focal changes. Capillary lumens were widely patent and contained a few erythrocytes and an occasional platelet (Fig. 1). In Stx1 treated mice, the capillary lumen was partially occluded by swollen endothelial cells, stagnant and

deformed erythrocytes in rouleaux formation and endothelial cell fragments (Fig. 2). There were degenerative changes in the endothelial cells with swelling of the peripheral cytoplasm, focal loss of endothelial fenestrations, cytoplasmic rarefaction and vacuolation, dilatation of rough endoplasmic reticulum and Golgi, and formation of intracytoplasmic myelin figures (Fig. 3). Numerous platelets appeared in the lumen, some of which showed signs of degeneration

with swelling and rarefaction (Fig. 4). Neutrophil polymorphs were only occasionally present (Fig. 2). Mesangial cells showed signs of degeneration including myelin figures.

Similar changes appeared in mice treated with Stx2. Capillary lumens were partially or completely obliterated by amorphous material, swollen endothelial cells and entrapped erythrocytes (Fig 5).

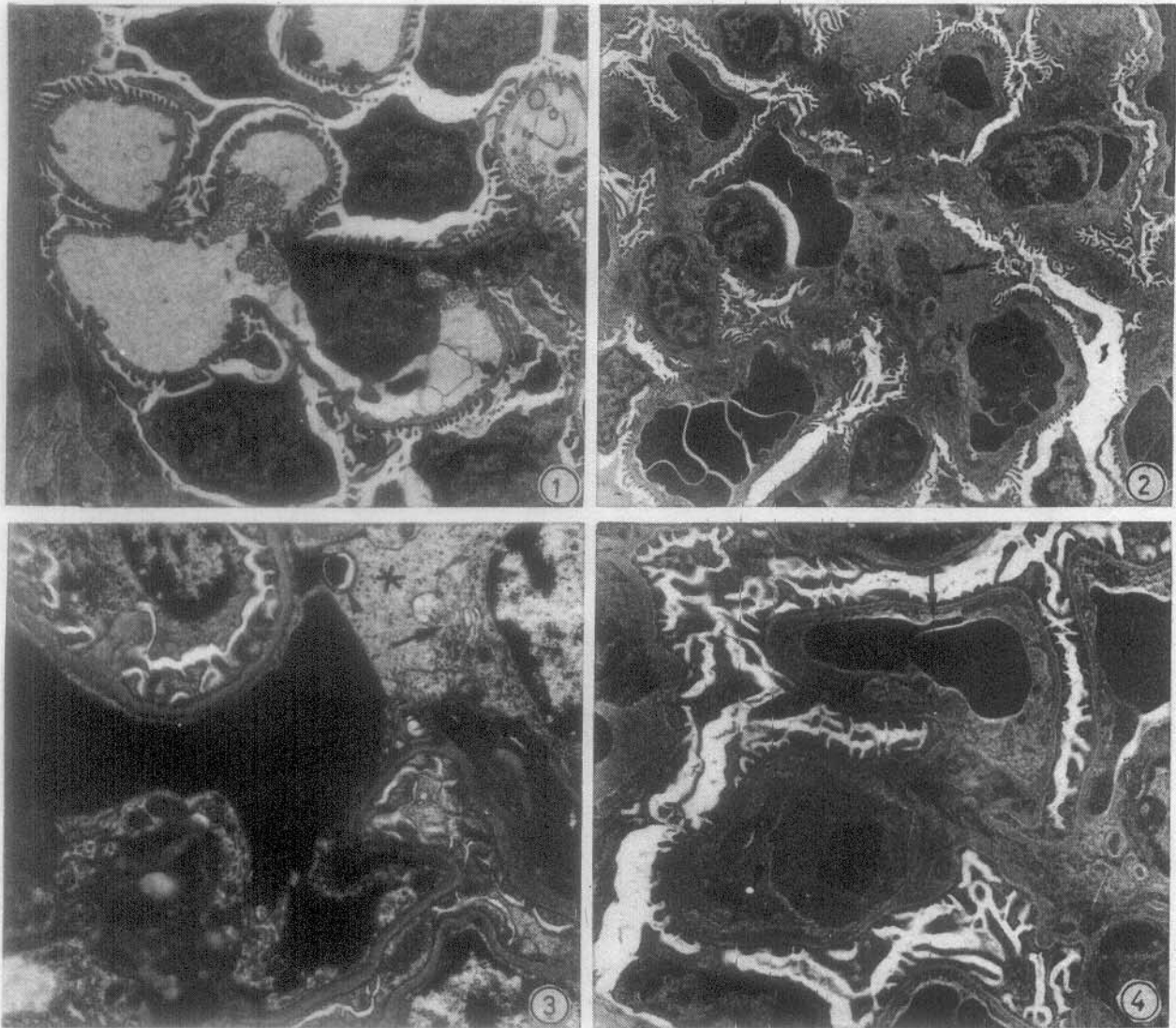


Fig. 1. Glomeruli from a saline treated control. Capillary endothelium is flat except in the region of the nucleus. The lumen is patent. Magnification X 4,400. **Fig. 2.** Glomeruli from a Stx1-treated mouse. Vascular congestion, swelling and fragmentation of the endothelial cell (arrow) is seen. N = neutrophil. Magnification X 2,700. **Fig. 3.** Endothelial cell swelling, rarefaction of cytoplasm (asterix), damage to cellular organelle (arrow) and myelin figures (arrowhead) are seen in the glomeruli of a mouse treated with Stx1. Magnification X 8,600. **Fig. 4.** Deformed erythrocytes (arrow) and adherent platelets (arrowhead) are found within the lumen of glomerular capillaries. Stx1-treated mouse. Magnification X 6,500.

Severely affected endothelial cells showed marked vacuolation and rarefaction of the cytoplasm and focal disruption of cell membrane (Fig. 6). Granular debris, platelets, fragments of endothelial cytoplasm and occasional fibrin tactoids were present in the capillary lumen (Fig. 7). There was damage to the erythrocytes with dehaemoglobinization, deformation and fragmentation of cells (Fig. 8). There was significant endothelial damage and vascular congestion in Stx1 and Stx2 treated mice, when compared to controls ($P < 0.005$). There was no

significant increase in neutrophils or platelets ($P = 0.05$) and no significant difference between the effects of Stx1 and Stx2 (Table).

Endothelial damage and platelet aggregation were also noted in peritubular capillaries. Tubular epithelial cells showed swollen mitochondria with loss of cristae, scattered dense bodies and clumping of nuclear chromatin (Fig. 9). There were large, clear cytoplasmic vacuoles in the arteriolar endothelium and smooth muscle cells (Fig. 10).

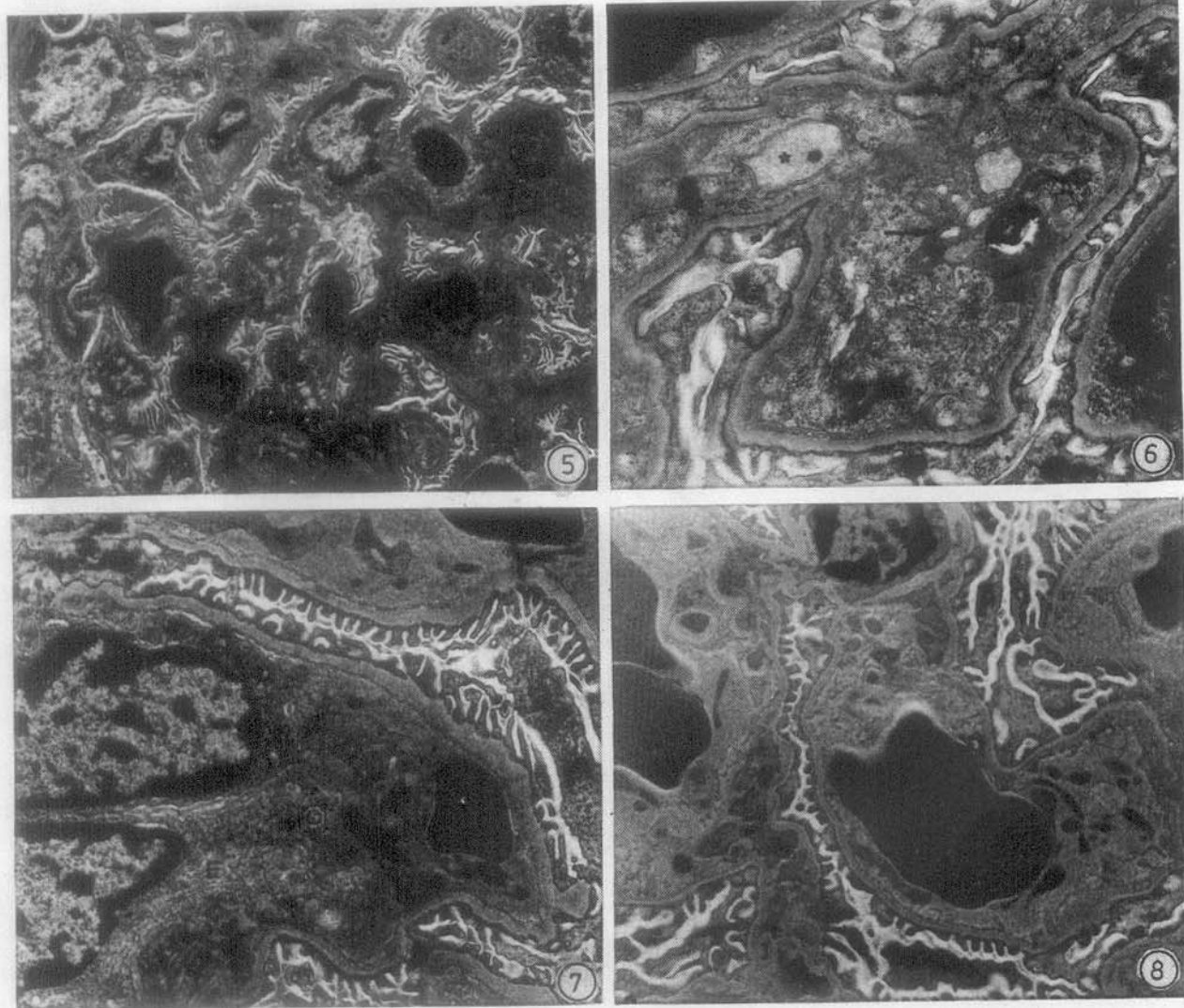


Fig. 5. Glomeruli from a Stx2-treated mouse. The capillary lumen is greatly compromised by swollen endothelial cells and amorphous material. Magnification X 2,600. **Fig. 6.** Vacuolation of endothelial cytoplasm (asterix) and disruption of cell membrane (arrowhead) in a glomerular capillary of Stx2-treated mouse. Cell fragments are seen in the lumen (arrow). Magnification X 17,400. **Fig. 7.** Glomeruli from a Stx2-treated mouse. Swollen endothelial cells (E) and fibrin tactoids (arrow head) obstruct the lumen. Magnification X 9,000. **Fig. 8.** Glomerular capillary from a Stx2-treated mouse containing many erythrocytic fragments (arrow head). Magnification X 6,300.

Table. Comparative frequency of lesions in glomerular capillaries of mice inoculated with saline, Stx1 or Stx2

	Saline (n=67) No. (%)	Stx1 (n=65) No (%)	Stx2 (n = 57) No. (%)
Endothelial swelling	01 (0.49)	43 (66.15)*	41 (71.93)*
Organelle damage	00 (0.00)	38 (58.46)*	31 (54.39)*
Cytoplasmic rarefaction	00 (0.00)	23 (35.38)*	26 (45.61)*
Cytoplasmic fragmentation	12 (17.90)	45 (69.23)*	35 (61.40)*
Cytoplasmic vacuolation	01 (1.49)	12 (18.46)*	26 (45.61)*
Any of the above	12 (17.90)	46 (70.77)*	48 (84.21)*
Vascular congestion	00 (0.00)	35 (53.85)*	35 (61.40)*
Neutrophil	00 (0.00)	06 (9.23)	01 (1.75)
Platelet	05 (7.46)	10 (15.34)	11 (19.30)

* $P < 0.001$ compared to saline controls; Stx1 - Shiga-like toxin I; Stx2-Shiga-like toxin II; n- number of glomerular capillary cross sections examined

Discussion

Intraperitoneal administration of endotoxin-free Stx1 and Stx2 produced systemic and neurologic signs in adult Swiss albino mice. Glomerular endothelial damage with swelling, vacuolation and lysis of endothelial cell and signs of early intravascular coagulation with accumulation of platelets and deposition of fibrin and arteriolar constriction with vacuolation of endothelial cells and smooth muscle were similar to the microangiopathy seen in renal biopsies of patients with HUS⁵. Earlier murine models of mice infected with shiga toxin-producing *Esch.coli* have shown thickening of glomerular capillary wall, congestion of glomerular capillaries and fibrin deposition in glomeruli, but no damage to endothelial cells^{10,12}. In the baboon model, swelling of glomerular endothelial cells in response to cell injury or activation was seen¹¹ but other signs of cell injury seen in our study *viz.*, organelle damage,

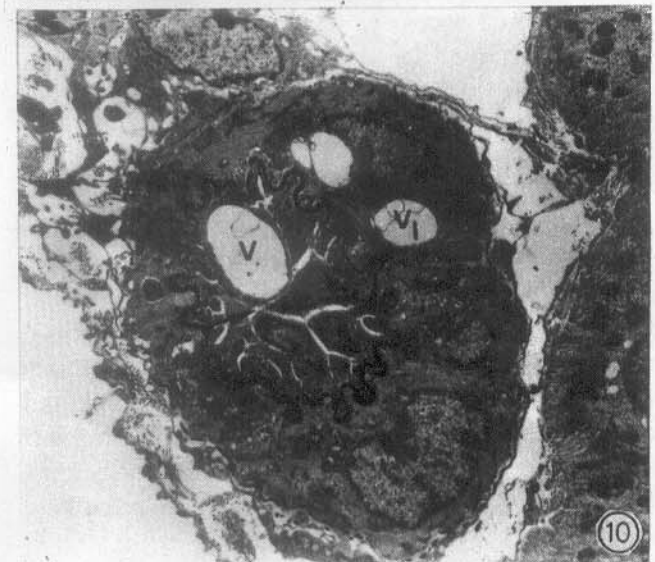
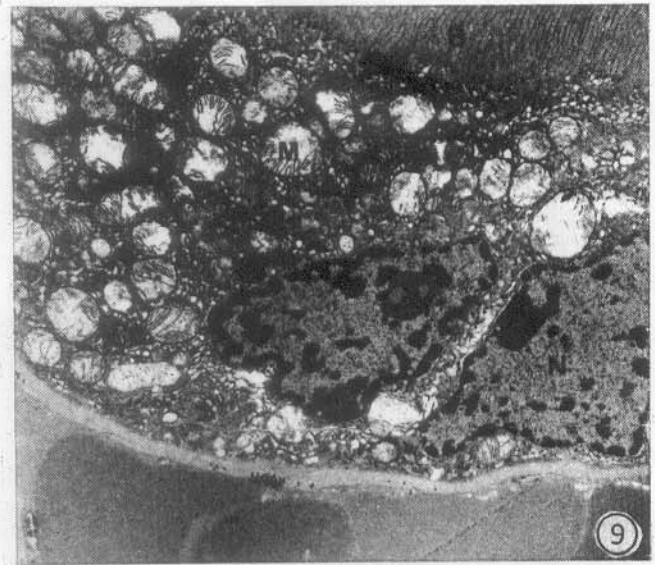


Fig 9. Tubular epithelial cell from a Stx2-treated mouse. B-brush border. M=swollen mitochondria with disruption of cristae. N = nucleus. Magnification X 6,200. **Fig. 10.** Constricted arteriole with narrow slit-like lumen (arrow). Degenerative vacuoles have formed in the endothelial cell (V) and smooth muscle cells of the media (V₁). Stx2-treated mouse. Magnification X 8,500.

cytoplasmic rarefaction, fragmentation and vacuolation of endothelial cells, have not been reported. Since endothelial damage is believed to be crucial in the pathogenesis of HUS^{6,7} and Stx producing *Esch.coli* are strongly associated with this complication^{3,9} it is necessary that an animal model used to study this relationship should show primary glomerular endothelial damage.

Microvascular endothelial cells contain high levels of globotriaosylceramide (Gb3), the functional receptor for Stx¹⁷. Endothelial damage may be a direct action of Stx on endothelial cells or may be compounded by the effect of proinflammatory cytokines like interleukin-1 (IL-1) and tumour necrosis factor (TNF α) from activated endothelial cells¹⁸. This microvascular endothelial injury results in a microthrombotic angiopathy that leads to a variable degree of renal ischaemia.

The sensitivity of Swiss albino mice to endothelial injury induced by Stx, is not seen in other strains of mice^{16,19}, possibly due to differences in receptor expression or variations in the inflammatory cascade triggered by Stx1 and Stx2. It is also possible that in models using oral route of infection¹⁹, a lower dose of toxin gained access to the circulation or the rate of accumulation of the toxin in the tissues was slow. In our study, toxin injected intraperitoneally was essentially in a high dose.

Our study supports the hypothesis that Stx is of direct pathogenic significance in the HUS and that the glomerular capillary endothelial cell is a primary target for the toxin. It provides an animal model relevant for studying the early vascular changes in response to Stx1 and Stx2. Further studies to clarify the reasons for this predisposition to glomerular and vascular damage in our model may aid in understanding the pathogenesis of Stx1 and Stx2 related disease and suggest possible therapeutic interventions.

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