INTRODUCTION

Snakebite is a serious health problem in Brazil. Of the 20,000 snakebite accidents reported per year, about 90% are attributed to the genus Bothrops. The most serious systemic effect and the most common complication in lethal cases is acute renal failure (ARF) secondary to acute tubular necrosis and occasionally glomerulonephritis. Glomerular changes in human victims of snakebite poisoning have attracted little attention, and there has been no systematic study of this action of snake venoms. Experimental studies of renal changes after injection of bothropic venom showed degenerative lesions of tubular cells and glomeruli, with mild proliferation of the mesangial matrix, glomerular congestion, and massive glomerular fibrin deposition immediately after venom injection. We observed a significant decrease in the glomerular filtration rate, an increase in the fractional urinary Na+ and K+ excretion, and significant histological changes in the tubular and glomerular structure after intravenous injection of Bothrops moojeni venom. The alterations observed by light microscopy include glomerulosclerosis characterized by hypercellularity, glomerular lopolulation with retraction of the capillary tuft, and condensed mesangial matrix-forming nodular lesions. The mean arterial pressure remained unchanged.

In this study, transmission and scanning electron microscopy (TEM/SEM) were used to characterize the glomerular alterations caused by B. moojeni venom in rats. The protein urinary excretion rates were also examined.

MATERIALS AND METHODS

Sample preparation. Male Wistar rats (200–300 g) were obtained from an established colony maintained by the University’s Central Animal House Service. Lyophilized B. moojeni venom was donated by the Instituto Butantan (São Paulo) and stored at −20°C. The rats were anesthetized with ether and received an intravenous injection of 0.4 mg/kg B. moojeni venom. The dose of 0.4 mg/kg was chosen because it had been used by Burdmann et al in their experimental model of venom-induced ARF. The venom was dissolved in 0.15 M NaCl immediately before use. One group of rats was injected with venom (V), and the control group received the vehicle, 0.15 M NaCl alone (S).

Measurement of proteinuria. The total urine protein excretion of the control group (n = 5) and of rats injected with B. moojeni venom was evaluated 5 hours (V, n = 5), 16 hours (V, n = 5), and 48 hours (V, n = 5) postinjection. At 8:00 a.m., each rat received a tap water load by gavage (5% of body weight) followed by a second load of equal volume 1 hour later. Twenty minutes after the second load, the rats were housed individually in metabolic cages, and spontaneously voided urine was collected over a 2 hour period. Urine samples were diluted in 1% sulfosalicylic acid, and the protein concentrations were determined spectrophotometrically at a wave length of 550 nm and compared with known albumin standards. Statistical analysis of the data was performed using nonparametric Kruskal-Wallis and Mann-Whitney tests. The significance level was set at P < 0.01.

SEM studies. Five hours (V, n = 2; S, n = 1) and 16 hours (V, n = 2; S, n = 1) after injection, the rats were anesthesized with sodium pentobarbital (Sagatal, 60 mg/kg i.p.) and perfused as in the TEM studies discussed later. After perfusion, renal cortical slices were immersed in Karnovsky’s solution (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) at 4°C overnight. After rinsing in phosphate buffer for 1 hour, the specimens were postfixed in buffered 1% OsO4 at 4°C in the dark for 2 hours and then immersed in a 2.3 M sucrose solution at 4°C overnight. The specimens were subsequently immersed for 30 min in liquid nitrogen and then fractured, washed in the same buffer, dehydrated in a graded acetone series, and critical-point dried (Balzers CPD 030). After identifying the fractured surface,
specimens were mounted on stubs, sputtered with gold (Balzers SCD 050) for 120 s, and examined and photographed with a Zeiss DSM 940A scanning electron microscope operated at 10 kV.

**TEM studies.** Five hours (V, n = 4; S, n = 2) and 16 hours (V, n = 4; S, n = 2) after injection, the rats were anesthetized with sodium pentobarbital (Sagatal®, 60 mg/kg, i.p.) and perfused in situ via the left ventricle using gravity flow, initially with 100 mL of saline containing 0.1% heparin followed by 2.5% glutaraldehyde in 0.1 M phosphate buffer for 20 min. After perfusion, the kidneys were removed, and cortical slices were cut into small pieces, which were immersed in the same fixative with 0.1% tannic acid and 5% sucrose for 3 hours at room temperature. After rinsing in a sugar-saline solution (0.15 M NaCl, 0.2 M sucrose), the specimens were postfixed with 1% OsO₄ at 4°C in the glucose-saline solution in the dark for 2 hours and then rinsed again in the glucose-saline solution. The samples were dehydrated in a graded ethanol series and embedded in Epon 812 resin at 60°C for 48 h. Thin sections (60–70 nm) were double-stained with uranyl acetate and lead citrate and were observed and photographed with a LEO 906 transmission electron microscope operated at 60 kV.

**RESULTS**

After 5 hours and 16 hours, four animals (two of each group) voided red-colored urine indicative of hematuria and/or hemoglobinuria. *B. moojeni* venom induced massive acute proteinuria, which was significantly different from control rats after 5 hours, 16 hours, and 48 hours (*P* < 0.01). These changes were short lived because they partially recovered by 48 hours (Figure 1) and were consistent with the SEM and TEM observations of drastic morphological alterations in the mesangium, podocytes and glomerular basement membrane (GBM).

The visceral epithelium of glomeruli from control rats injected with 0.15 M NaCl solution had a normal ultrastructure as seen by SEM. The podocytes of the epithelium closely covered all of the outer surface of the glomerular capillary loops. The primary processes of the podocytes emerged from the nuclear portion and surrounded the capillary loops. An interdigitating primary process derived from an adjacent podocyte always occurred between two primary processes of the same contiguous podocyte. The primary processes branched, giving rise to secondary and tertiary processes or pedicels. The terminal processes or pedicels interdigitated closely with similar processes from adjacent podocytes, leaving narrow filtration slits between them (Figure 2 A and B). The bulging surface of the podocyte bodies also showed occasional short pedicel-like microprojections (see Figure 2 A and B). This elaborate epithelium was anchored to the outer surface of the underlying thick GBM surrounding the glomerular endothelium.

Five hours (Figure 2 C) and 16 hours (Figure 2 D) after a venom dose of 0.4 mg/kg, the glomerular visceral epithelium lost its peculiar cohesive appearance, and the tips of the pedicels projected into the Bowman's space. Upright pedicels appeared as long, thin, club-ended projections emerging from

**FIGURE 1.** Time course of the proteinuria after the injection of *B. moojeni* snake venom (0.4 mg/kg i.v.) compared with saline-treated (S) rats. The columns represent the mean ± SEM. *P* = 0.01 compared with S.

**FIGURE 2.** Scanning electron micrographs of glomeruli. A. Three-dimensional organization of the outer surface of podocytes (p) surrounding capillaries of a renal glomerulus in a control rat (× 4,800, bar = 2 μm). B. Detail of the entwined processes of the podocytes of a control glomerulus. Note the primary (1) and secondary (2) processes and the pedicels (arrowhead) among which filtration slits can be seen (arrow) (× 9,900, bar = 1 μm). C. A damaged glomerulus 5 hours after venom injection in which the swollen podocytes exhibit villous transformation involving highly thin and branched filamentous pedicles with a chaotic appearance and no cohesive arrangement (× 4,800, bar = 2 μm). D. View 16 hours after envenomation; note the numerous wavy microprojections representing noninterdigitating pedicels and the loss of filtration slits. Note also the club-shaped distal ends of the pedicels. (× 17,600, bar = 0.5 μm.)
the podocytes. Consequently, the filtration slits disappeared, and there was obvious impairment of the filtration barrier. In light microscopy, this feature appeared as glomerular lobulation. Another change observed was the increased number of microprojections arising from the nuclear portion of podocytes in venom-treated rats. This increase in microprojections is characteristic of a disorder known as villous glomerular transformation.

TEM showed that in saline-treated rats the filtration barrier retained its normal organization of interdigitating pedicels bridged by the filtration slit membrane, the GBM, and the thin endothelium with fenestrations (Figure 3 A and B). However, this regular arrangement of the podocyte foot processes disappeared almost completely 5 hours (Figure 3 C and D) and 16 hours (Figure 3 E and F) after venom injection. In the presence of venom, the podocytes showed intense simplification, with loss of the filtration slits and the appearance of wide, irregular profiles at the terminal ends of the pedicels.

There was a reduction in the complexity of cell–cell connections as a result of podocyte simplification or foot process effacement, which ranged from partial retraction of the foot processes to total disappearance of the usual interdigitated pattern. In addition, the cytoplasm of pedicels showed electron-dense irregular masses (Figures 3 D–F and 4 C) and the number of endothelial fenestrations decreased (Figures 3 C and D and 4 A). The regular width and homogeneous appearance of the GBM was also lost, with this membrane now showing discontinuities (Figure 3 D) or becoming narrower and denser (Figure 3 F) than in the controls.

Additionally, the podocyty processes showed several alterations, including accumulation of absorption droplets in the cell’s lysosomal system (Figure 4 A), irregular amorphous masses of granular material (Figure 4 C), pseudocyst formations (Figure 4 A–C), an increasing polyribosome population (Figure 4 E and F), and a peculiar arrangement of protein electrondense deposits within greatly enlarged rough endoplasmic reticulum cisternae (Figure 4 E and F) that were not found in the controls. Degenerative changes were also seen in the intraglomerular mesangium. The lesions began with local dissolution of the mesangial matrix or mesangiolysis as well as local protrusion of the mesangial cells into glomerular capillaries, which, together with expanded glomerular endothelial cell bodies, could cause occlusion of the capillaries (Figure 5). Other prominent features in glomeruli 5 hours and 16 hours after venom injection were capillary ballooning and the eventual formation of microaneurysms (Figure 6).

**DISCUSSION**

The results of this study show that in rats *B. moojeni* venom causes proteinuria and ultrastructural changes in the visceral epithelium and glomerular capillaries tufts compatible with
the renal dysfunction described elsewhere.\(^9\) Significant proteinuria occurred 5 hours, 16 hours, and 48 hours after venom. However, the level of proteinuria had recovered considerably after 48 hours, indicating that the process was reversible. Proteinuria has been associated invariably with effacement of the podocyte foot processes of glomerular epithelia\(^10\) and lysosomal accumulation.\(^11\) In agreement with this, we found visceral epithelial changes that included podocyte simplification as well as lysosomal clusters accumulated in podocyte endings. In addition, the appearance of electron-dense clumps of amorphous bodies in the cytoplasm of the remaining processes, the formation of pseudocysts, and the loss of normal slit-diaphragm integrity and orientation were associated with glomerular dysfunction. These features were seen in both SEM and TEM. Arakawa and Tokunaga\(^12\) showed that the loss of foot processes is caused by their withdrawal into the cell body and not by the fusion of adjacent processes to form a synctium. Foot process effacement or simplification would represent a reduction in the complexity of the usual interdigitating pattern of cell–cell connections and an obvious diminution of filtration capacity.

Club-ended pedicels (by SEM) and enlarged processes (by TEM) were seen resting over the GBM and contained clumps of electron-dense material. An explanation for such changes described elsewhere\(^13\) includes podocyte fixation to the GBM via broad, sheet-like processes that contained a highly organized network of cytoskeletal proteins formed by microfilaments regularly cross-linked to dense bodies, with prominent expression of \(\alpha\)-actinin. These findings have been interpreted as indicative of an adaptive change in the cell in which the rearrangement and hypertrophy of the contractile apparatus would reinforce podocyte adherence to the GBM.\(^13\) Similar alterations have been found in human glomerulonephritis associated with nephrotic syndrome\(^14,15\) and in nephrosis induced by puromycin aminonucleosides, which are toxic to visceral epithelial cells.\(^11,16,17\) Changes in the state of cytoskeletal aggregation are associated with decreased fibrillar attachment of the epithelium to the GBM and with the onset of massive proteinuria.\(^16,18,19\) Our results reproduced these abnormal features and provided evidence for a toxic effect of \textit{B. moojeni} venom on visceral epithelial cells.

To understand how \textit{B. moojeni} venom may induce such podocytic alterations, it must be recalled that most of the glomerular F actin is concentrated in the foot process of

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**FIGURE 5.** Partial view of renal glomeruli 16 hours after venom injection. A and inset a are electron micrographs showing the cell body of a mesangial cell and components of the mesangial matrix, both occluding the glomerular capillary. A, left side. A capillary with several bulbous projections (\(\bigstar\)), which are less electrondense than endothelium, can be seen. B. The glomerular capillaries occluded by the cell bodies of endothelial (e) and mesangial (m) cell. Some cytoplasmic projections from mesangial cells imbricate with the endothelium. Note the hypertrophied Golgi apparatus (arrowheads) of the mesangial cell. C. Diapedesis of a mesangial cell through the constricted lumen of a capillary. (A, \(\times 6,681\), bar = 1.5 \(\mu\)m; inset a, \(\times 9,000\), bar = 1 \(\mu\)m; B, \(\times 11,150\), bar = 1 \(\mu\)m; C, \(\times 14,400\), bar = 1 \(\mu\)m.)

**FIGURE 6.** Two partial views of glomeruli 5 hours (B) and 16 hours (A) after venom injection. A. The capillary (c1) forms a complete ring (microaneurysm) with fragments of mesangium (arrows); in the urinary space (us), there are numerous microprojections and/or segments of wavy pedicels. Inset a. The decreased density of the mesangial matrix. B. The abnormal location of a mesangial cell (m1) beneath the endothelium (e); another mesangial cell (m2) is in its usual location between the capillary loops. Note that capillary represents a microaneurysm, and its lumen contains endothelial and mesangial cell fragments. c, capillary; b, basal lamina; co, collagen bundle. (A, \(\times 4,008\), bar = 3 \(\mu\)m; a, \(\times 7,515\), bar = 1 \(\mu\)m; B, \(\times 5,344\), bar = 2 \(\mu\)m.)
podocytes, and that these densely-packed cytoskeletal filaments are associated with the ability to maintain and/or generate significant changes in podocyte shape as well as to provide support for the foot processes. Consequently, alterations in the state of F actin filament polymerization responsible for anchoring the pedicels to the GBM would alter the function of the filtration barrier.

Andrews reported that the treatment of glomeruli in vitro with cytochalasin B (a drug that inhibits the contraction of actin filaments) changed the shape of podocyte foot processes and thereby altered the number of open filtration rifts. These alterations may have been involved in the changes in the ultrafiltration coefficient and, consequently, in the rate of glomerular filtration. Because the glomerular and renal tubule epithelial cells are strategically interposed between the extra- and intramembrane, they are potential targets for numerous nephrotoxic agents, and the podocytes are the first structure of the nephron to come in contact with circulating venom.

The proteolytic and phospholipase A₂ activities of Bothrops venoms have important cytotoxic effects in many cell types, and could contribute to the nephrotoxicity seen in the current study. A hypothetical cytotoxin-like mechanism of action has been proposed for bothropic myotoxins acting on cultured cells, liposomes, and skeletal muscle cells in vivo. After binding to an unidentified site on the cell plasma membrane, the myotoxins would penetrate the bilayer through hydrophobic interactions, destabilizing the membrane and causing impaired permeability to ions and macromolecules. A prominent calcium influx would probably be the most important consequence of membrane disturbance and would be responsible for the onset of a variety of destructive mechanisms such as cytoskeletal alterations, mitochondrial damage, and the activation of calcium-dependent proteases and endogenous phospholipases, which, in turn, would account for the cellular damage.

The ultrastructural alterations induced by the snake venom in the trilaminar structure of the GBM suggest the involvement of physicoelectrostatic barrier sites. Podocytes and endothelial cells retain heparin sulfate in the lamina rara externa and interna of the GBM, and the feet processes are coated with sialoproteins that contribute to the anionic charges of the filtration barrier. This ionic barrier of glomerular filtration was shown to be destroyed by Vipera russelli venom in isolated rat kidneys.

Several proteins, including type IV collagen, laminin, entactin, and heparin sulfate proteoglycan are common components of basement membranes, although there are important tissue-to-tissue differences in the relative proportion. The strong attachment of podocytes to the GBM is based on α₅β₁ integrin-fibronectin–laminin interactions. Baramova et al demonstrated the ability of crotalic hemorrhagin to proteolytically degrade the major components of the basal lamina and extracellular matrix, such as laminin, fibronectin, type IV collagen, entactin/nidogen, and gelatins. Ohsaka et al studied the action of Trimeresurus flavoviridis snake venom on isolated GBM in vitro and demonstrated that proteins (or peptides) and carbohydrates were released from the GBM by venom fractions. Because the GBM is formed by the union of the basal lamina of the capillary endothelium and the visceral epithelium, and the endothelium of these capillaries is fenestrated, thereby facilitating contact between venom hemorrhagins and the components of the filtration lamina, B. moojeni venom could digest some of these components. In addition, proteolytic enzymes in B. moojeni venom could alter the connections between laminin and the integrins of the pedicels, releasing the latter from their anchorage to the GBM. Structural and functional disturbances of the GBM in venom-treated rats could indirectly cause podocyte RE cisternae dilatation with accumulation of proteinaceous material. This, in turn, could result in an anomalous rise in protein synthesis to replace the GBM and/or podocyte cytoskeletal constituents.

Another characteristic ultrastructural alteration induced by B. moojeni venom was a rapid onset of mesangiolysis, capillary ballooning, and formation of microaneurysms. The first report of mesangiolysis-like lesions after experimental envenomation with snake (Crotalus adamanteus) venom was published in 1909. Agkistrodon halys snake venom also induces mesangiolysis, and this damage has been reported after bites from the habu snake Trimeresurus flavoviridis. The high proteolytic activity of the latter venom was thought to cause most of the damage to the glomerular mesangium and could also account for B. moojeni venom-induced mesangiolysis.

The formation of glomerular microaneurysms is poorly understood, although a failure in the supporting system of the glomerular tuft because of mesangiolysis is usually the underlying cause. In addition to the mesangium, the podocytes and GBM also provide a structural basis for capillary tuft support, and all undoubtedly participate in protecting the capillaries from ballooning or microaneurysm formation otherwise seen after mesangiolysis.

In summary, the current study demonstrates for the first time that glomerular changes are responsible for proteinuria and that both contribute to the nephrotoxicity in ARF caused by the intravenous administration of B. moojeni venom. It seems likely that mesangiolysis, microaneurysm formation, and pedicel damage are a consequence of the high proteolytic and PLA₂ activities of this venom. Studies are being designed to identify the biological phenomena responsible for such ultrastructural changes.

Acknowledgments: We thank Gustavo Henrique da Silva for technical assistance, Dr. Stephen Hyslop (Universidade Estadual de Campinas/UNICAMP) for revising the language of an earlier version of this article, Dr. Luiz Antonio Ribeiro de Moura (Universidade Federal de São Paulo/UNIFESP-EPM) and Dr. Fábio Bucaretchi (Universidade Estadual de Campinas/UNICAMP) for helpful suggestions, and Dr. Elliot Watanabe Kitajima (EASALQ-USP) for use of the SEM.

Financial support: This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Fundação de Amparo à Pesquisa do Estado de São Paulo (Proc. 93/0995-5, 98/00340-4, 98/00341-0), Fundo de Apoio ao Ensino e à Pesquisa (FAEP—UNICAMP), and Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico (Proc. 522131/95-6).

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