Focal Mesangial Proliferative Glomerulonephritis in the Rat Caused by Habu Snake Venom

A Morphologic Study

Victoria Cattell, MRC Path, and John W. B. Bradfield, MRC Path, PhD

A new model of focal mesangial proliferative glomerulonephritis in the rat has been produced by intravenous habu snake venom. Glomerulonephritis developed in 70% of rats surviving the first 6 hours after venom administration. The earliest ultrastructural change (10 minutes after venom) was the presence of loose platelet aggregates and free granules in the capillary lumen and mesangium. This was followed by dissolution of the matrix and endothelial damage. Between 4 and 24 hours, a characteristic focal and segmental ballooned lesion of glomerular capillaries developed. In these lesions, from 3 days onwards a segmental mesangial proliferation occurred, which persisted until sacrifice at 21 days. (Am J Pathol 87:511–524, 1977)

THE GLOMERULAR CHANGES which follow intravenous injection of habu (*Trimeresurus flavoviridis*) snake venom have been called *mesangiolysis*.[1,2] This term denotes ultrastructural changes in the mesangium which begin 6 hours after injection as a decrease in density of the matrix with mesangial cell vacuolation and culminate in complete disintegration of the mesangium with cystic dilatation of capillary loops. Mesangiolysis has also been observed after administration of venoms from other viperous snakes,[3] and some of these features occur in Masugi nephritis [4] and in irradiation nephritis.[5]

Our experiments were done to investigate two things: a) to clarify the earliest morphologic changes in the glomerulus after habu venom and b) to see if the response to early mesangial injury might be mesangial cell proliferation. This paper describes how segmental capillary loop injury leads to a segmental mesangial hypercellularity. The kinetics of this proliferative response will be described elsewhere.[6]

Materials and Methods

Animals

Highly inbred male Lewis rats weighing 180 to 350 g were used.

From the Department of Pathology, St. Mary's Hospital Medical School, London, England.
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Address reprint requests to Dr. Victoria Cattell, Wellcome Laboratories of Experimental Pathology, St. Mary's Hospital Medical School, London, W2 1PG, England.

Snake Venom

Lyophilized venom from the habu pit viper, *Trimeresurus flavoviridis*, (from the Japan Snake Institute) was dissolved in phosphate-buffered saline at a concentration of 1 mg/ml.

Production of Glomerulonephritis

Habu venom in a single dose of 2 to 4 mg/kg was injected into the lateral tail vein of rats anesthetized with ether.

Biopsy Technique

Biopsies were performed at intervals of 10, 30, and 60 minutes; 2, 4, 6, and 24 hours; 3, 5, 7, and 21 days under ether anesthesia. The kidney was exposed via a midline abdominal incision and mobilized by stripping the anterior peritoneum. A cortical wedge was obtained using a scapel blade, and hemostasis of the wound was achieved by packing with Surgical Oxidised Cellulose (Ethicon Ltd., Edinburgh, Scotland). Nephrectomies were performed using the same approach.

Preparation of Tissues for Examination

Cortical renal tissue obtained at biopsy or nephrectomy was divided into two pieces. One piece was fixed in Bouin's solution and processed for light microscopy. The second piece, for electron microscopy, was diced into 2-mm cubes. Half this material was fixed in buffered glutaraldehyde at 4 C for standard electron microscopic processing, and the other half fixed in buffered 2% osmium tetroxide for rapid processing. For light microscopy, pieces in Bouin's solution were transferred at 24 hours into 70% alcohol before embedding in paraffin wax. Four-micron sections were stained with hematoxylin and eosin or periodic acid–Schiff reagent. For electron microscopy, tissue fixed in buffered glutaraldehyde was postfixed in buffered 2% osmium tetroxide (pH 7.4) at 4 C for 1 hour, dehydrated, and embedded in epoxy resin. Tissue fixed in osmium tetroxide was processed by a rapid processing schedule. Tissue was dehydrated for 25 minutes in ascending grades of acetone and alcohol and then embedded in 1:1 acetone and Epon for 10 minutes, Epon accelerator mixture for a further 10 minutes, and then in Epon.

Sections (0.5 μ) were cut, stained with Azure II, and examined by light microscopy to select glomeruli for electron microscopy. Ultrathin sections showing silver-gray interference colours were cut, stained with Reynold's lead citrate, and examined using a Philips EM 300 electron microscope.

Results

Survival

Immediately following a single intravenous injection of habu snake venom, all rats developed respiratory distress, and some developed hematuria within 6 hours. There was wide variation in mortality between different experimental groups; this is documented in Table 1. Rats surviving the first 6 hours after injection were still alive after 21 days, despite an initial period of poor peripheral circulation as shown by the development of gangrenous patches on the tips of the ears.

Table 1—Twenty-Four Hour Survival in Rats Given Intravenous Habu Venom

Experiment	Dose	No. of Animals	Survivors
1	2 mg/kg	4	4
2	2 mg/kg	5	5
3	2 mg/kg	3	3
Total		12	12
4	4 mg/kg	10	6
5	4 mg/kg	6	5
6	4 mg/kg	5	4
7	4 mg/kg	12	10
8	4 mg/kg	12	8
9	4 mg/kg	4	2
10	4 mg/kg	19	4
11	4 mg/kg	4	4
Total		72	43
12	4 mg/kg × 2	4	3

Incidence of Lesions

Forty of 58 survivors developed segmental cystic glomerular lesions which progressed to segmental mesangial hypercellularity (Table 2).

Morphology

Nonsurvivors

Rats dying within 6 hours had generalized capillary congestion in all major organs and focal hemorrhages in the lungs and gut.

Survivors

Light Microscopy. In 18 of 58 rats the kidneys were normal. Forty animals developed glomerulonephritis. The sequence of morphologic changes in these animals was as follows: 2 hours after venom the glomerular capillaries were congested. Between 6 and 24 hours, some glomeruli developed a segmental ballooning of capillary loops (Figure 1). The number of glomeruli affected in individual animals varied between 3 and 30%. Inside the cystic loops there were increased numbers of polymorphonuclear leukocytes, red cells, and variable amounts of fibrin. No fibrin was seen outside the capillaries in Bowman's space. The unaffected segments of the glomeruli appeared completely normal. On Day 3 there was a slight increase in cellularity in abnormal loops and mitotic figures were frequent. By Day 7, as shown in Figure 2, these loops had completely filled with mononuclear cells. No crescents were seen. At sacrifice

Table 2—Histologic Results in 12 Different Experiments in Rats Surviving for 24 Hours After Receiving Intravenous Habu Venom

Dose	No. of Animals	Histology							
		24 hours				3–31 days			
		0	+	++	+++	0	+	++	+++
2 mg/kg	5	5				5			
2 mg/kg	4		1	2	1		1	2	1
2 mg/kg	3		1	2			*		
Total	12	5	2	4	1	5	1	2	1
4 mg/kg	6		4	2			4	2	
4 mg/kg	5		1	3	1		*		
4 mg/kg	4		*				3	1	
4 mg/kg	2	2				2			
4 mg/kg	4	3	1			3	1		
4 mg/kg	10	2	1	4	3	2	1	4	3
4 mg/kg	8	5		3		5		3	
4 mg/kg	4	0	1	1	2		*		
Total	43	12	8	13	6	12	9	10	3
4 mg/kg × 2	3	1		2		1		2	

* No biopsy.
Percentage of glomeruli involved: + = 0 to 10, ++ 10 to 20, +++ = 20 to 30.

(after 21 days), segmental hypercellularity was still present as were mitoses in proliferative glomeruli.

Electron Microscopy. STAGE 1 (10 minutes–4 hours). The most striking ultrastructural feature in early biopsies was the presence of loose platelet aggregates in many glomerular capillary lumens. The platelets showed pseudopod formation with a central shift of organelles. Small numbers of platelets had completely degranulated. An unexpected finding was the large number of free membrane-bound granules in the plasma around platelets, under the endothelium, and in clusters in the mesangial matrix (Figures 4 and 5). Similar structures were occasionally seen in the canalicular system of intact platelets. There was no granule release from the peripheral platelet membrane. Small areas of glomerular basement membrane and mesangium were exposed to lumen where gaps had developed in the endothelium (Figure 4).

There was no definite evidence of mesangial cell necrosis. Some mesangial cells had abnormally dilated rough endoplasmic reticulum, with extension of cytoplasmic processes into the capillary lumen, but most were normal. Around the clusters of platelet granules the mesangial matrix appeared less dense ("edematous") (Figures 5 and 6). Occasional polymorphonuclear leukocytes were marginated against the endothelium.

STAGE 2 (4 hours–3 days). The earliest fully developed segmental cystic lesions were seen at 4 hours (Figure 7). In affected glomeruli, the bal-

looned capillaries occupied the greater part of the glomerular space, with the remaining capillary loops appearing completely normal. The glomerular basement membrane around cystic loops was unbroken. Epithelial cells showed patchy foot process fusion. There were areas of endothelial loss, where platelets and occasional red cells were in contact with glomerular basement membrane. "Knots" of mesangial cells and matrix protruded at intervals from the inside of cystic loops. Some mesangial cells had platelets closely applied to their plasma membranes (Figure 8). Careful examination of many abnormal glomeruli failed to provide definite evidence of mesangial cell destruction. There was only one mesangial region containing cellular debris which might have originated from mesangial cell necrosis. From 4 hours onwards, cystic loops contained increasing amounts of fibrin and platelet masses.

STAGE 3 (3 to 21 days). Segmental mesangial hypercellularity with patent peripheral capillary lumens lined by intact endothelium was first seen 3 days after venom (Figure 9). The uniform population of mononuclear cells were mesangial cells as judged by position and ultrastructural features (Figure 10). They were embedded in mesangial matrix and had cytoplasmic processes which rarely formed junctions with adjacent cells. They had irregularly indented nuclei, moderate amounts of rough endoplasmic reticulum, prominent Golgi bodies, and dense cytoplasm. The hypercellular areas did not contain cells with macrophage or lymphocytic characteristics. There was no epithelial cell proliferation.

Discussion

Habu snake venom is very toxic, and in the rat renal damage only occurred at nearly lethal doses (Table 1). There was considerable variation in both mortality and the incidence of glomerulonephritis between different experimental groups (Table 2).

Nature of the Initial Damage and the Production of Cystic Glomerular Lesions

The biochemistry of snake venom has revealed that each venom contains a multitude of active principles, including phospholipases and proteases capable of cellular destruction.[7] Direct cytopathic effects on vascular endothelium have been demonstrated *in vivo*[8-10] and on cell monolayers *in vitro*.[11,12] Habu venom causes extensive local hemorrhagic necrosis in clinical cases of snake bite.[13] The initial endothelial and mesangial injury in this model could be the result of the direct action of a component of the venom.

However, the findings of platelets and free granules in glomerular capillaries as the earliest ultrastructural abnormality suggests that the

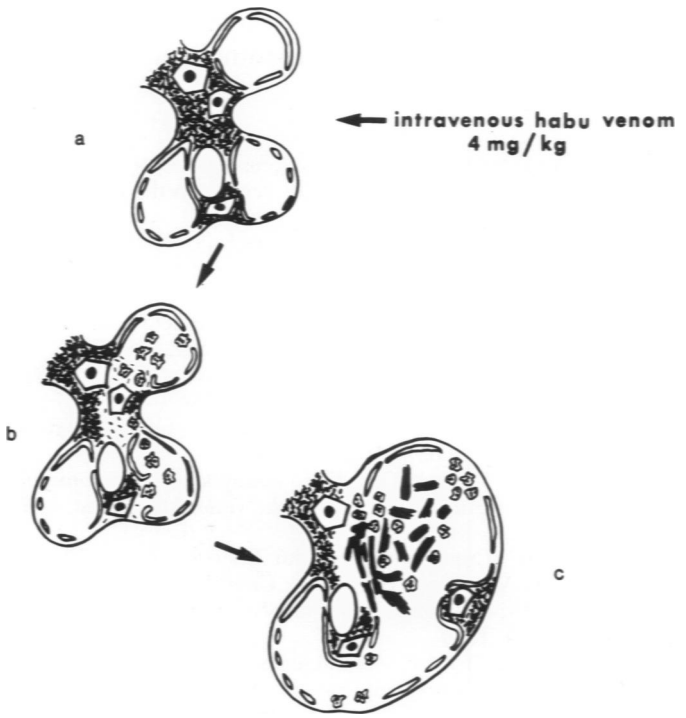
initial damage could be the result of a venom-induced platelet release reaction. A variety of snake venoms, including that of *Trimeresurus purpuromaculata* have been shown to induce platelet aggregation and release *in vitro*,[14] and thrombocytopenia is a common feature of venom administration.[15,16] Dissolution of the mesangial matrix and endothelium in contact with platelet granules supports this hypothesis. The observation of free platelet granules is of interest. This phenomenon was also found in glomerular capillaries following thrombin administration [17] and in the early phase of hyperacute rejection.[18] *In vitro*, platelet aggregation induced by cationic polypeptides is followed by extrusion of intact platelet granules.[19] The presence of intact granules inside the open canalicular system with granules of varying density in the surrounding plasma may indicate a similar mechanism in venom-induced platelet release. The exact mechanism by which the initial injury leads to the characteristic cystic loop lesion is still unclear. The mesangial cell disintegration previously described by Suzuki, Churg, and their colleagues [2] as a component of mesangiolysis was not confirmed in our experiments. A more likely explanation is that the breakdown of mesangial matrix and endothelial cytoplasm causes loss of axial support leading to coalescence of adjacent capillary loops (see Text-figure 1).

Mesangial Proliferation

By extending the period of observation after administration of habu venom, we have shown the development of a segmental glomerular hypercellularity, which has not previously been documented. Although reports on the effects of snake venoms on the kidney are few, proliferative glomerulonephritis has followed 2 clinical cases of snake bite poisoning.[20] Further, mesangial proliferation has been shown to follow "mesangiolytic" changes in rabbit Masugi nephritis.[4]

Further experiments on this model have confirmed that the hypercellularity is due to local mesangial cell proliferation.[6] The stimulus which induces mesangial cell proliferation is not known. However, it has two important characteristics: a) it acts locally within the area of the glomerulus initially damaged and b) the effect outlasts the stimulus because, after transplantation into a normal syngeneic recipient, proliferation still occurs.[6] This stimulus could either be mesangial injury itself, or other associated events such as platelet, polymorphonuclear leukocyte, or fibrin accumulation in damaged areas. Each of these latter agents has been postulated as a mediator of glomerular proliferation.

The demonstration of mitogenic agents in the products of the platelet release reaction,[21,22] together with our observations on platelet granule



TEXT-FIGURE 1A—Three capillary lumens are lined by flattened endothelial cytoplasm and supported centrally by mesangial matrix (*dark stippled areas*) in which are embedded three mesangial cells. B—Following injection of habu venom, loose platelet aggregates form in the capillaries. Products released from platelets dissolve areas of the matrix and injure endothelium. The mesangial cells remain intact. C—Mesangial tissue "snaps;" some remains centrally and some forms knots at the periphery of the lesion. The three loops coalesce into one large ballooned capillary loop. Platelets and fibrin strands have accumulated in the lumen.

accumulation at sites of glomerular injury, might indicate that the platelet reaction in this model is the source of the proliferative stimulus. A focal proliferative glomerulonephritis has been reported following ADP-induced platelet aggregation in rabbits.[23] The role of platelets in the production of glomerulonephritis has been recently reviewed.[24]

The contribution of fibrin to endocapillary glomerular proliferation has not been defined. However, defibrination will inhibit extracapillary proliferation (crescent formation) in experimental antiglomerular basement membrane nephritis [25] and chronic serum sickness nephritis.[26] In view of the suggested role of fibrin spillage in crescent formation, it may be relevant that in this model fibrin was confined within the capillary loops and extracapillary proliferation did not occur.

We have induced a focal and segmental proliferative glomerulonephritis by the administration of habu venom. Experiments are in progress

to see whether this new model will provide a means of analyzing the relative roles of platelets, coagulation, and immunologic agents in the pathogenesis of glomerulonephritis.

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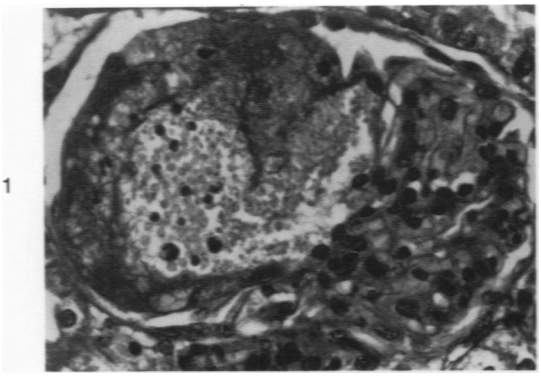
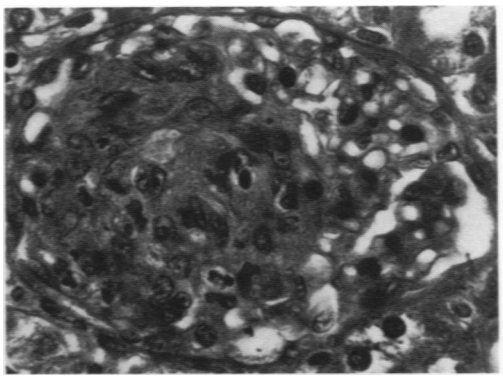
Dr. Bradfield's present address is Division of Nuclear Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD 21205.

[*Illustrations follow*]





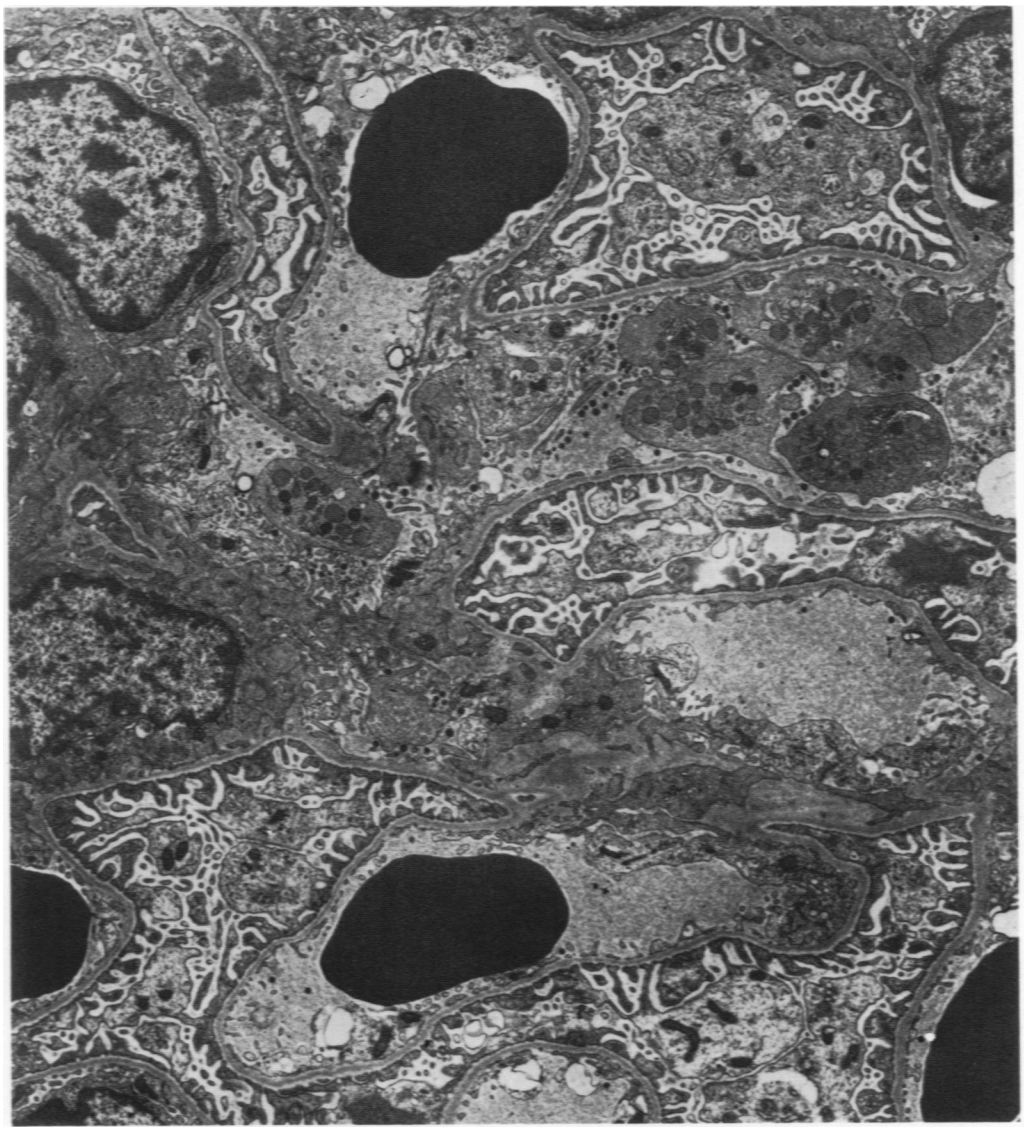
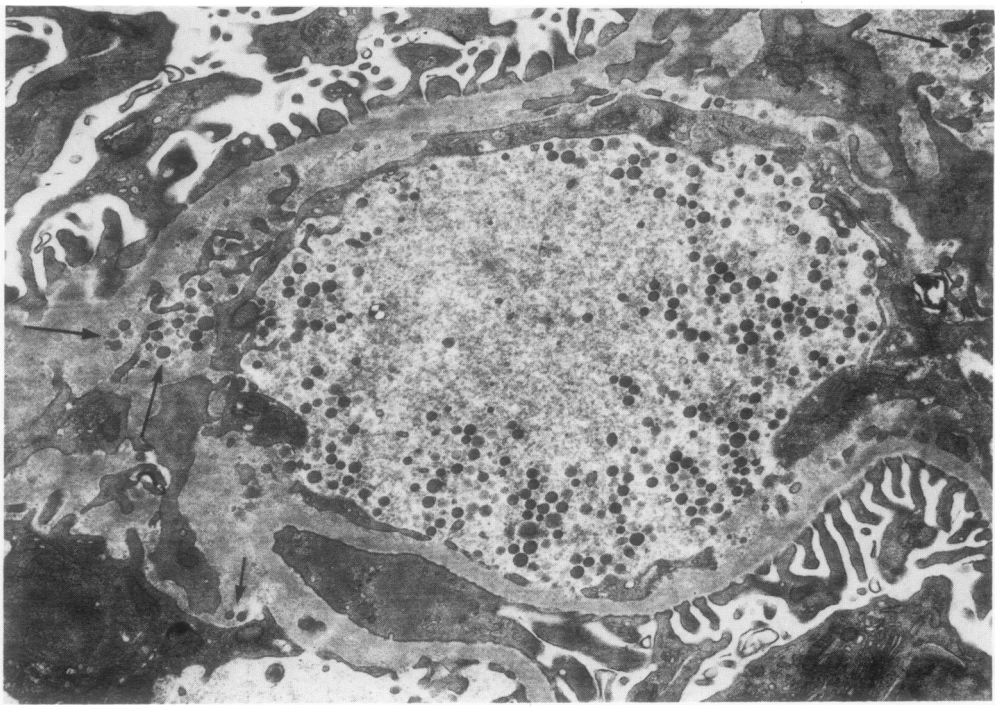
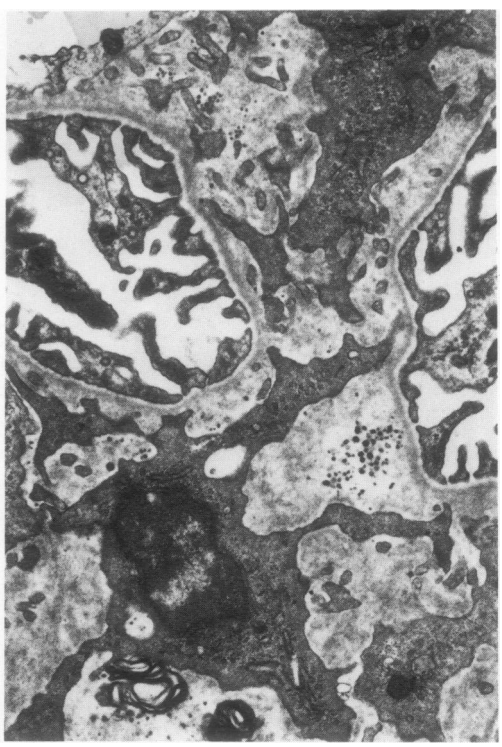


Figure 1—Segmental glomerular lesion 24 hours after injection of venom. The cystic loop contains red cells, fibrin, and polymorphonuclear leukocytes. (H&E, × 400) **Figure 2**—Segmental glomerular proliferation 5 days after venom (H&E, × 460). **Figure 3**—Electron micrograph showing platelet aggregates and free granules in capillaries 30 minutes after venom (× 6900).





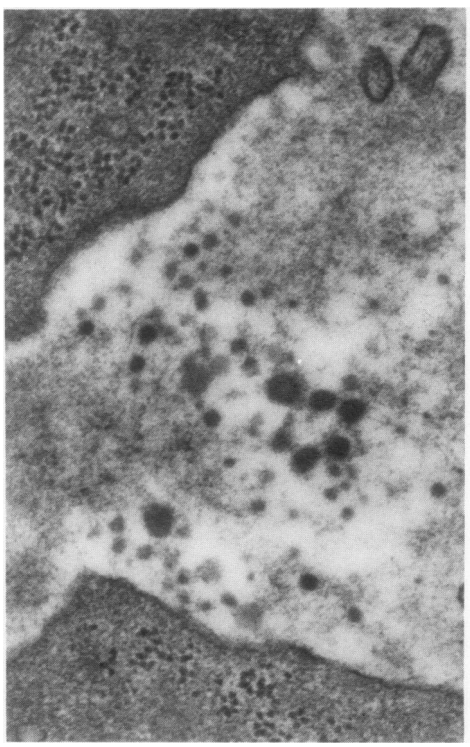
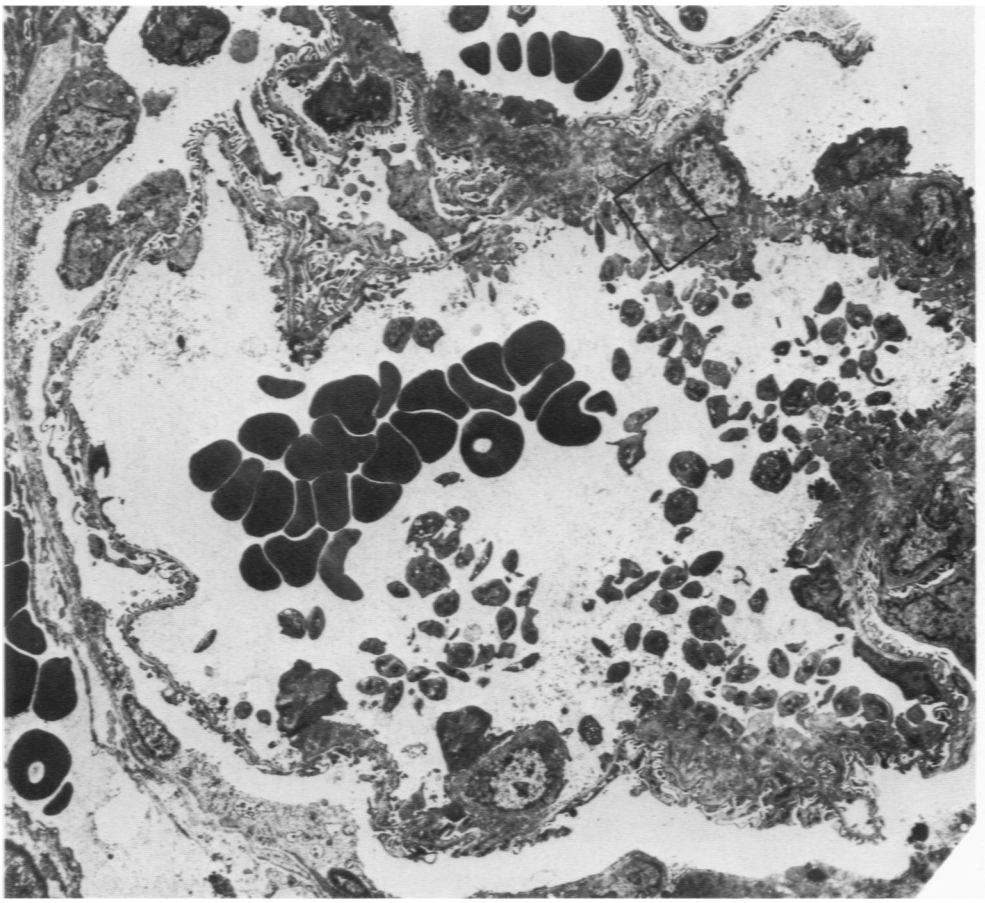


Figure 4—Electron micrograph of glomerular capillary 2 hours after venom. Free granules can be seen in the lumen, subendothelium, and mesangium (*arrows*). There is focal endothelial loss. (× 12,000) **Figure 5**—Electron micrograph showing clusters of platelet granules throughout the mesangium. The matrix around the granules has become electron lucent. (× 13,700) **Figure 6**—Higher power of the granule cluster in Figure 5. "Mesangiolysis" has developed around the granules. (× 61,500)



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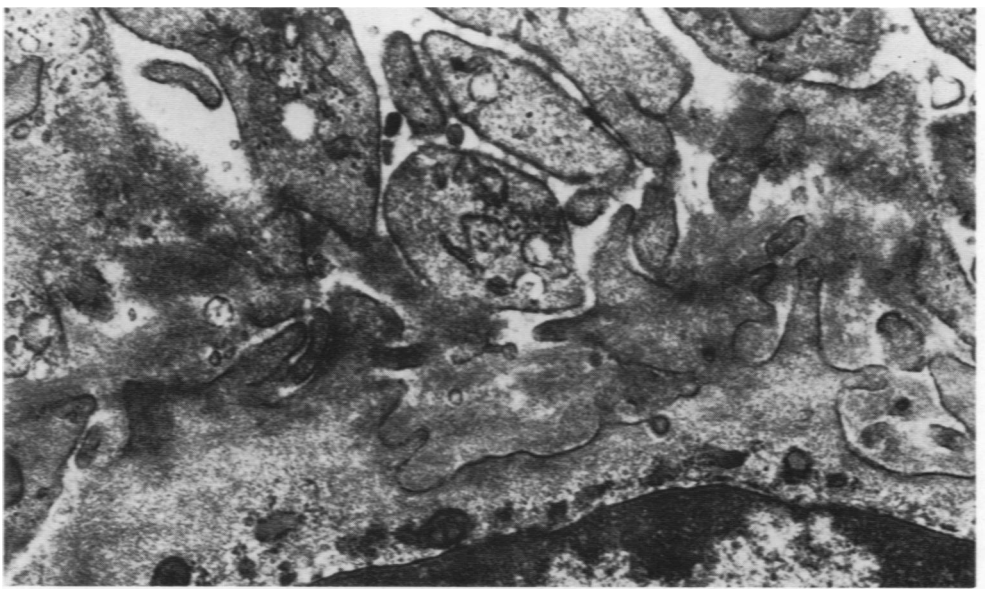

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Figure 7—Cystic glomerular loop 24 hours after venom. The glomerular basement membrane is intact. Knots of mesangial tissue persist around the periphery. In areas of endothelial loss, platelets are adhering to mesangium and basement membranes. (× 1920) **Figure 8**—Higher power of mesangial area boxed in Figure 7. Platelets are adhering to the surface of an intact mesangial cell. (× 31,800)

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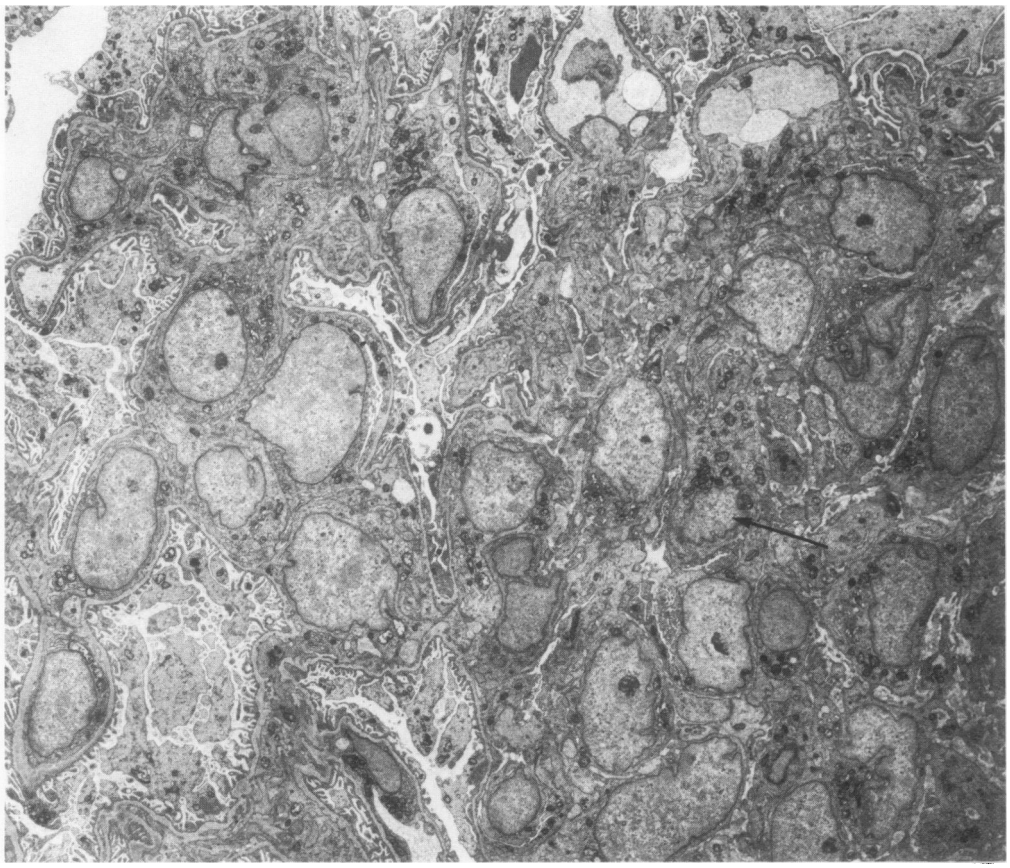

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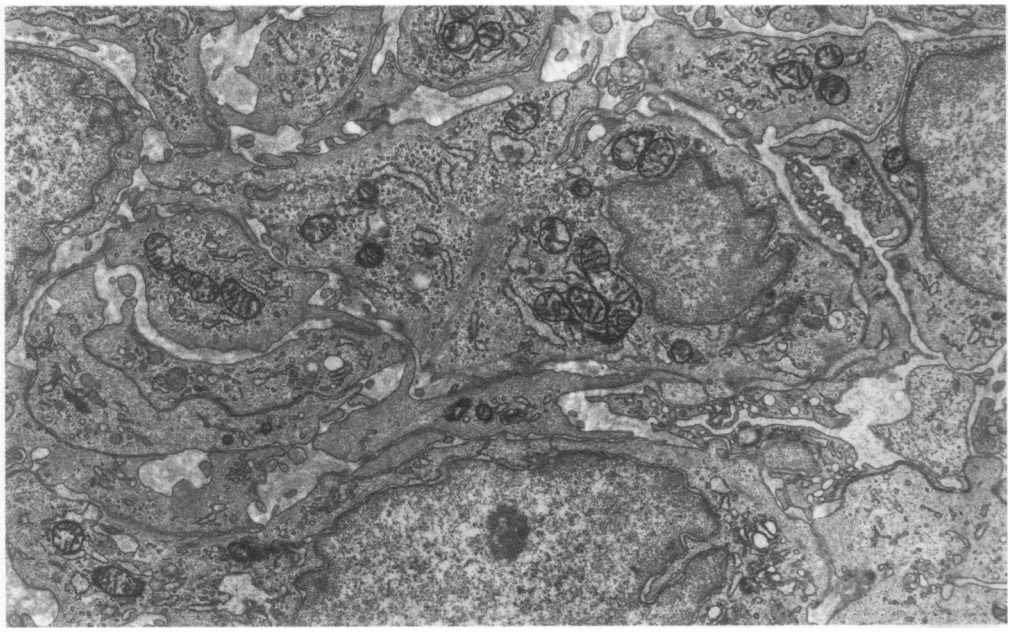

Figure 9—In the right half of the picture a single ballooned glomerular capillary contains large numbers of mesangial cells. A patent capillary lumen lined by endothelium can be seen at the periphery of the expanded mesangium. (× 2000) **Figure 10**—Higher power of mesangial cell arrowed in Figure 9. The cell is embedded in mesangial matrix. It has an irregular nuclear outline and cytoplasmic processes extending between adjacent cells. (× 5150)