Br. J. exp. Path (1986) 67, 1–12

Pathogenesis of myonecrosis induced by coral snake (*Micrurus nigrocinctus*) venom in mice

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Received for publication 11 December 1984
Accepted for publication 21 July 1985

Summary. The mode by which coral snake (*Micrurus nigrocinctus*) venom affects skeletal muscle was studied using a combined approach. The venom induced early functional and structural alterations in the plasma membrane of muscle cells, suggesting that sarcolemma is the primary site of action of this venom. This was shown by the presence of wedge-shaped ('delta') lesions at the periphery of the cells, as well as by focal disruptions in the continuity of plasma membrane as early as 15 min after envenomation. After this initial alteration the rest of the organelles were severely affected. Myofilaments were hypercontracted leaving, as a consequence, areas of overstretched myofibrils as well as empty spaces. Eventually, myofilaments formed dense, clumped masses in which the striated structure was totally lost. At 24 h, myofilaments were still disorganized but they presented a more hyaline and homogeneous appearance. As early as 15 and 30 min mitochondria were swollen; later, by 1, 3 and 24 h, they showed further alterations such as the presence of dense intracristal spaces and vesiculated cristae, as well as disruption in the integrity of their membranes. Sarcoplasmic reticulum was dilated and disorganized into many small vesicles randomly distributed throughout the cellular space. Moreover, the venom induced a rapid decrease in muscle levels of creatine and creatine-kinase (CK) and a calcium influx. Since the rates of efflux of creatine and CK were similar, it is suggested that the lesions produced in the membrane are large enough to allow the escape of these two molecules. As corroboration of the severe myotoxic effect, envenomated mice excreted reddish urine containing large quantities of myoglobin. Skeletal muscle cells are more susceptible to the action of the venom than erythrocytes, since coral snake venom induced only a mild direct haemolytic effect *in vitro* and haemolysis is not a significant effect *in vivo*. *M. nigrocinctus* venom induced a drastic increase in plasma levels of lactate dehydrogenase. Isozymes LDH-3, LDH-4, and LDH-5 increased markedly, suggesting that the systemic pathology of coral snake envenoming may be more complex than previously thought.

Keywords: myonecrosis, coral snake venom, *Micrurus nigrocinctus*, myoglobinuria, haemolysis

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Many snake venoms induce skeletal muscle necrosis in humans and laboratory animals (Homma & Tu 1971; Gutiérrez & Chaves 1980; Mebs *et al.* 1983). It has been demonstrated that some elapid venoms contain potent myotoxins, such as notexin and taipoxin, isolated from the venoms of the Australian elapids *Notechis scutatus* and *Oxyranus scutellatus*, respectively (Harris *et al.* 1975; Harris & Maltin 1982). Studies on the pathogenesis of myonecrosis induced by snake venoms are important, not only to obtain a better understanding of envenomations, but also to use these venoms as tools to approach more general aspects of muscle pathology.

Coral snakes are the American representatives of the family Elapidae; they comprise more than 50 species (Roze 1970). It has been known that coral snake venoms induce neurotoxic and cardiovascular effects (Jiménez-Porras *et al.* 1973; Brazil *et al.* 1978). Recently, Gutiérrez *et al.* (1983) demonstrated that they also exert a potent myotoxic activity in mice, suggesting the possibility that myotoxicity might be relevant in envenomings after coral snake bites.

The present study was designed to obtain a better understanding of the pathogenesis of myonecrosis induced after experimental inoculations of venom of the Central American coral snake *Micrurus nigrocinctus*. Particular attention was given to the detection of early pathological changes in skeletal muscle cells.

Materials and methods

Venom. The venom used was a pool obtained from more than 60 specimens of *Micrurus nigrocinctus* collected in Costa Rica.

Histology and ultrastructure. Groups of four mice (20–24 g) were injected im in the right thigh with venom (30 μg) in 0.1 ml of phosphate-buffered saline solution (pH 7.2). At several time intervals after injection (15 min, 30 min, 1 h, 3 h and 24 h) muscle samples were obtained from the envenomated thigh, cut into small pieces, and processed for light and electron microscopy as previously described (Arroyo & Gutiérrez 1981). Thick sections were stained with toluidine blue and thin sections with uranyl acetate and lead citrate; the latter were examined in an Hitachi HU-12A electron microscope. Control mice were injected with 0.1 ml of saline solution.

Changes in wet weight. Groups of four mice (20–24 g) were injected im in the right gastrocnemius with venom (30 μg). At different time intervals (30 min, 1 h and 3 h) mice were killed and both gastrocnemius muscles were obtained and weighed. Changes in wet weight of envenomated muscle were expressed as percentages, taking as 100% the weight of the contralateral gastrocnemius.

Changes in creatine kinase (CK) and creatine contents of skeletal muscle. Groups of four mice (20–40 g) were injected im in the right gastrocnemius with venom (30 μg). At several time intervals (30 min, 1 h and 3 h) they were killed and both gastrocnemius muscles were obtained. They were immediately homogenized in 5 ml of phosphate-buffered saline containing 0.1% Triton X-100. Homogenates were then centrifuged at 9000 *g* and the supernatants collected. An aliquot was diluted 35 times with distilled water and the CK content was estimated using the Sigma Kit Number 520 (Sigma Chemical Co., St Louis, MO, USA). The creatine content of another aliquot was determined (Sigma Kit Number 520). CK and creatine contents were expressed as percentages, taking as 100% the values of the controlateral gastrocnemius.

Changes in calcium levels of muscle. Groups of four mice (20–24 g) were injected im in the right thigh with venom (30 μg). One and 3 h later, animals were killed and changes in cytoplasmic calcium levels of muscle fibres determined using the metallochromic calcium indicator Arsenazo III, according to

Gutiérrez *et al.* (1984). Approximately 50 mg of muscle were transferred to a vial containing: Arsenazo III 0.1 mM, digitonin 200 μg/ml, NaCl 140 mM, KCL 5 mM, and imidazole 10 mM (pH 7.0) in a total volume of 2.0 ml. After exactly 1 min, 1.0 ml of the supernatant was transferred to a separate vial. The absorbance of the solution at 675 and 685 nm was read in a Varian-Techtron spectrophotometer. Calcium levels were directly proportional to the differential absorbance at 675–685 nm. As controls, groups of four mice were injected with 0.1 ml of saline solution; at 1 and 3 h pieces of muscle were obtained and calcium levels determined as described above.

Detection of myoglobin in plasma and urine. Groups of four mice (20–24 g) were injected im with venom (30 μg). One and 3 h later, they were killed and samples of urine and plasma obtained and analysed electrophoretically in 1% agar (dissolved in Tris-EDTA-borate buffer, 0.06 M, pH 9.1). Electrophoreses were performed using barbital buffer, 0.025 M, pH 8.6, according to Neremberg (1975). Standards of myoglobin and haemoglobin were run in each determination. Haeme-containing proteins were detected by adding a colour reagent made of benzidine and hydrogen peroxide. Under these experimental conditions, myoglobin and haemoglobin can be separated due to their different electrophoretic mobilities.

Changes in lactate dehydrogenase (LDH) isozymes. Groups of four mice (20–24 g) were injected im in the thigh with venom (30 μg). At 1, 3 and 6 h blood samples were collected from the tail into heparinized capillary tubes, and electrophoresis of plasma was performed in order to detect changes in LDH isozymes, utilizing the Sigma Kit No. 705-EP (Sigma Chemical Co., St Louis, MO, USA).

Myotoxic activity in vitro. The extensor digitorum longus muscle of guinea pigs was dissected out, attached to a capillary tube, and was incubated at 37°C in a test tube containing 5 ml of physiological salt solution bubbled with 95% O_2: 5% CO_2. The solution contained coral snake venom (50 μg/ml). At 30 min, 1 h, and 2 h samples of the solution were obtained and concentrations of CK and creatine were determined using Sigma Kit No. 520 (Sigma Chemical Co., St Louis, MO, USA). Control experiments, from which venom was excluded, were incubated and sampled at the same time intervals; control values were subtracted from values obtained in experiments with venom. Release of CK and creatine were expressed as percentages, taking as 100% the release of these molecules in muscle incubated for 2 h with physiological salt solution containing 1% Triton X-100. Experiments were repeated from four to six times.

Direct haemolysis in vitro. Different concentrations of coral snake venom were incubated with mouse erythrocytes previously washed five times with saline solution and diluted to a concentration of 2.5%. Tubes were incubated for 1 h at 37°C and centrifuged; absorbance of the supernatant was recorded at 541 nm, and haemolysis was expressed as a percentage, taking as 100% the absorbance of the supernatant of erythrocytes incubated with distilled water. In order to test the influence of calcium in direct haemolysis, some experiments were performed with calcium (2 mM) whereas in others the assay mixture contained EDTA (2 mM) and no calcium.

Results

Light microscopy

Muscles from animals injected with saline were histologically normal. In venom-injected mice, skeletal muscle damage was observed as early as 15 min after envenoming. The first alterations in muscle fibres were characterized by focal, wedge-shaped areas of degeneration at the periphery of the cells (Fig. 1). These alterations are very similar to 'delta lesions' described in other muscle

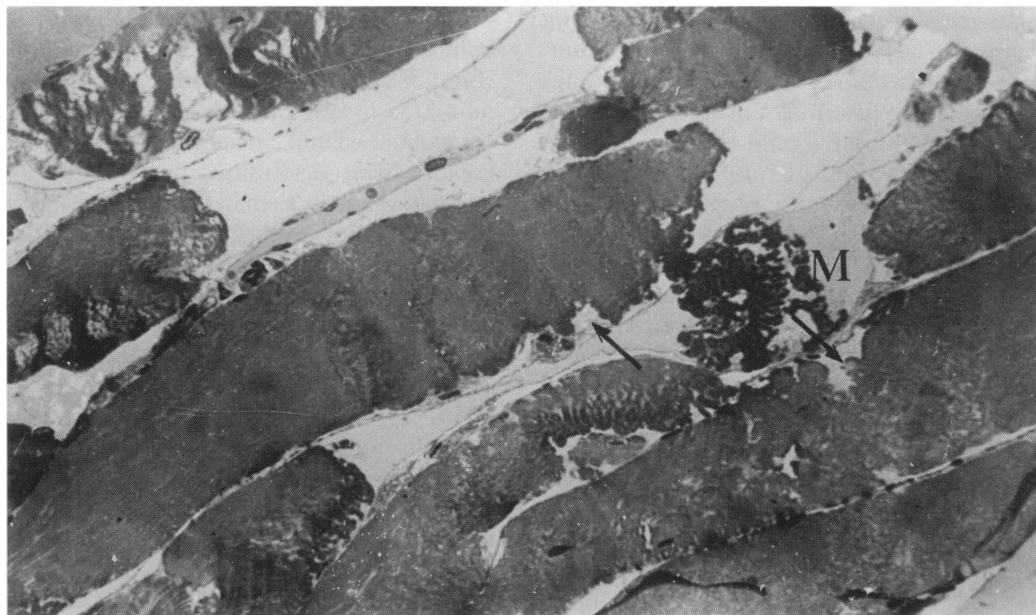

Fig. 1. Photomicrograph of skeletal muscle taken 15 min after venom injection. Peripheral, wedge-shaped lesions ('delta lesions') are observed (arrows) in some cells, whereas others show a more advanced degeneration, with clumped masses of myofilaments (M). × 360.

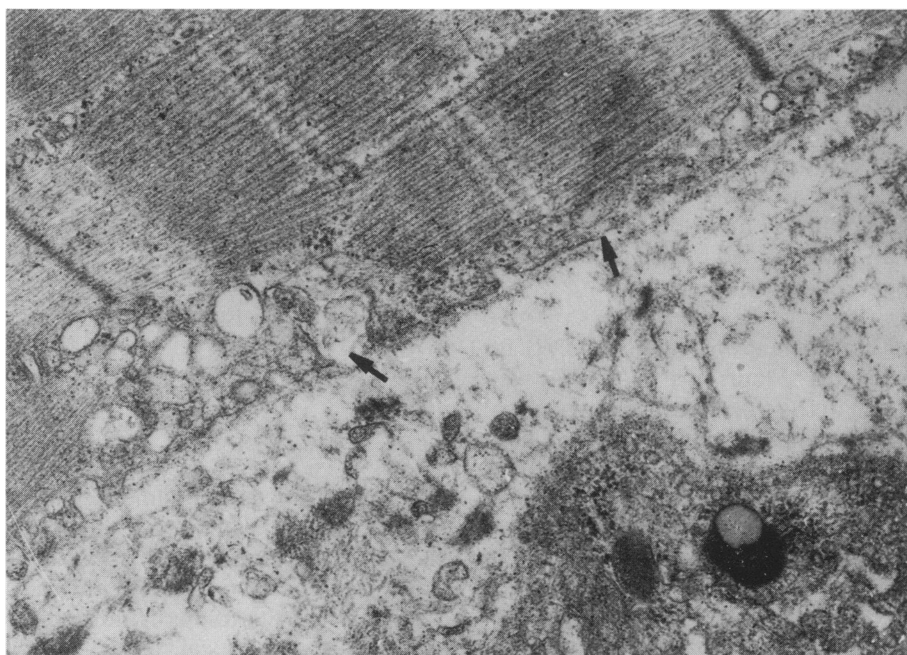

Fig. 2. Electron micrograph of skeletal muscle 15 min after injection of *M. nigrocinctus* venom. Portion of a cell with interruptions in the integrity of the sarcolemma (arrows). The myofibrils have not yet been affected. Notice the presence of an intact basal lamina. × 35 000.

pathologies such as Duchenne muscular dystrophy (eg Mokri & Engel 1975). At 15 min, a few cells were in a more advanced stage of degeneration and their cytoplasm was disorganized into amorphous, dense clumps. By 1 h and 3 h there was widespread muscle damage in which cells were drastically affected, with clumped myofibrils alternating with empty spaces in the cytoplasm. At 24 h necrotic fibres had a more hyaline appearance and distribution of myofibrillar material was more homogeneous; at this period, necrotic cells had been invaded by phagocytes. *M. nigroinctus* venom did not induce vascular alterations such as haemorrhage and thrombosis.

Electron microscopy

The ultrastructure of control muscle was normal in all samples examined. On the other hand, in venom-injected muscle the plasma membrane was damaged soon after envenoming. At 15 min many cells had focal interruptions in the integrity of the sarcolemma (Fig. 2). Since some cells had alterations only at the plasma membrane; this indicates that the sarcolemma was altered before any other organelle. Also at 15 min many cells showed peripheral areas of degeneration in which the plasma membrane was disrupted or totally absent. Beneath these focal sarcolemmal alterations there were localized areas of disorganization of myofibrils (Fig. 3). In these areas, the basal lamina remained intact at the periphery of the cell (Fig. 3). The first alterations observed in myofilaments, at 15 min, were characterized by areas of hypercontraction alternating with areas of overstretched myofibrils (Fig. 4). In some cells, the areas of hypercontraction alternated with empty spaces in the cytoplasm. By 30 min myofibrillar material was disorganized into amorphous, dense clumps in which the structure was totally lost.

At 30 min, 1 h and 3 h many skeletal muscle fibres were irreversibly injured. Their

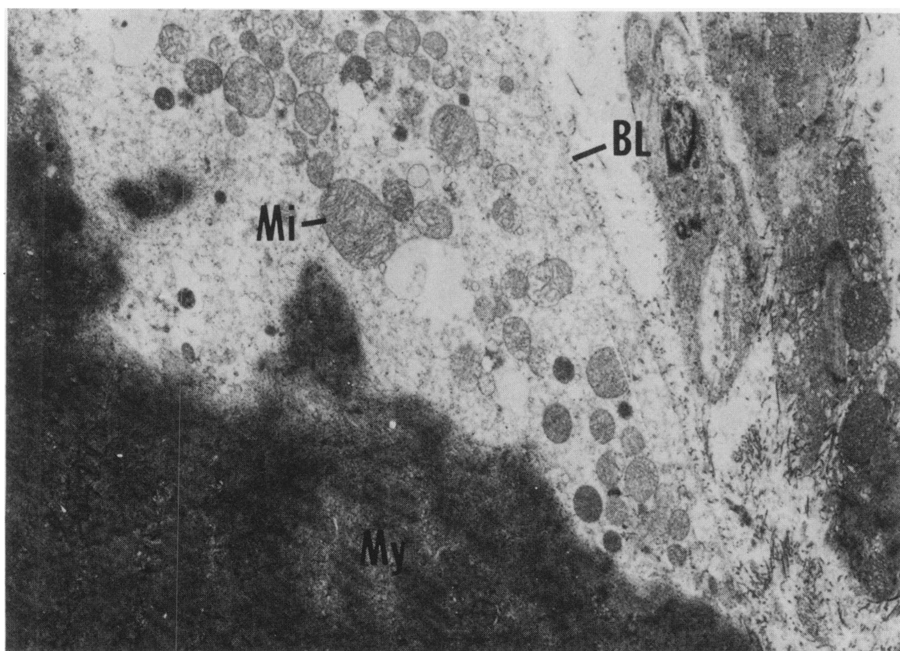

Fig. 3. Electron micrograph of skeletal muscle taken 15 min after injection of *M. nigrocinctus* venom. Peripheral area of a necrotic fibre in which the sarcolemma is absent although the basal lamina is intact (BL). Myofilaments (My) are disorganized into a dense clump and mitochondria (Mi) are swollen. × 5200.

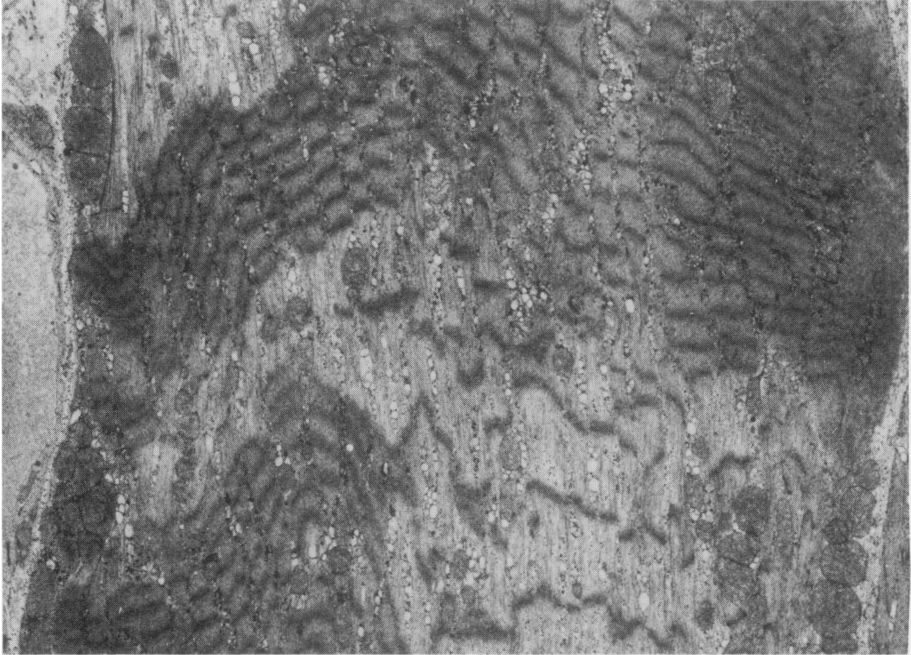

Fig. 4. Electron micrograph of skeletal muscle. Changes in myofibrils 15 min after envenomation. Early alterations in which areas of hypercontraction alternate with overstretched portions. This stage precedes the formation of clumped masses of myofilaments. ×4500.

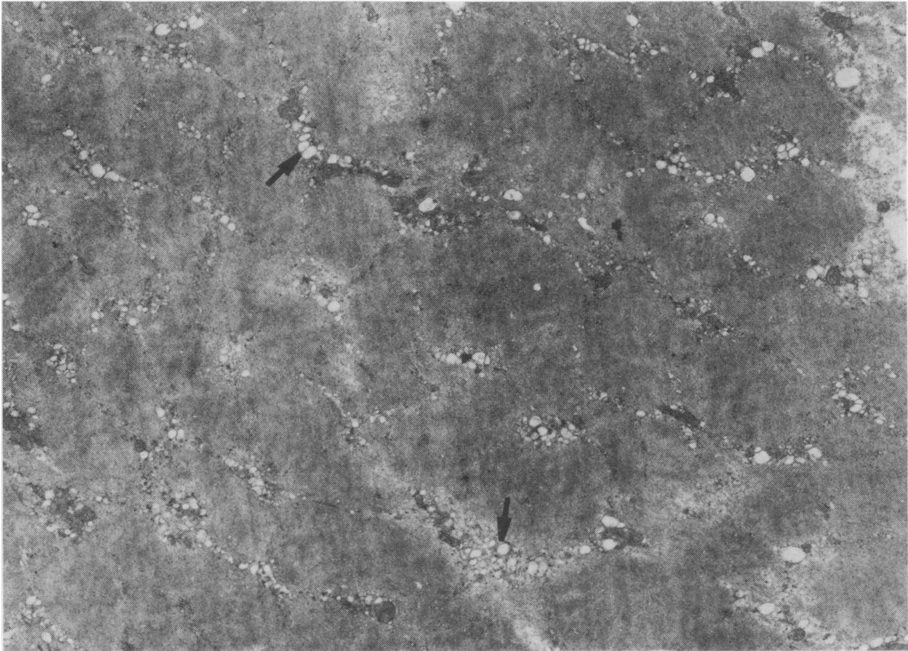

Fig. 5. Electron micrograph of skeletal muscle 24 h after injection of *M. nigrocinctus*. Myofibrillar material is disorganized to form a hyaline, homogeneous mass of myofilaments in which the normal striated pattern has been lost. Sarcoplasmic reticulum (arrow) shows moderate dilation. × 5200.

plasma membranes were missing although the basal lamina was apparently intact. Myofilaments were clumped into dense masses that left many empty spaces in the cytoplasm. In some areas, myofibrillar-like material was observed in the interstitial space. Twenty-four hours after envenoming the disposition of the myofilaments had changed to a less dense, more hyaline pattern with a more homogeneous distribution in the cellular space (Fig. 5). Sarcoplasmic reticulum was severely affected by 15 min after envenoming, and there were many small vesicles distributed throughout the cellular space. Furthermore, T-tubules were not observed in necrotic muscle, indicating that they also were disrupted. Mitochondria showed sequential damage; by 15 and 30 min they were swollen but many of them retained their internal structure, but after longer time periods (1 h, 3 h and 24 h) they were severely damaged. Many were swollen with dense intracristal spaces and vesiculated cristae; some contained only one membrane and were disrupted (Fig. 6). Nuclei also were severely affected with dense chromatin and disruption of the nuclear envelope in many cells. In samples obtained 24 h after envenoming there was an abundant phagocytic cell infiltrate in the interstitial space and inside necrotic muscle fibres. *M. nigrocinctus* venom did not affect the morphology of capillary vessels.

Changes in wet weight, CK, creatine and calcium

There was a moderate increase in wet weight of envenomated gastrocnemius, reaching a value of $112 \pm 3\%$ ($n=4$) at 3 h (Fig. 7). A parallel decrease in muscle creatine and CK was observed (Fig. 7) from as early as 30 min, thus reflecting functional alterations in the plasma membrane. By 3 h, CK and creatine levels were $35.7 \pm 8\%$ ($n=4$) and $34.1 \pm 2\%$ ($n=4$), respectively, by comparison with controls. Moreover, a prominent

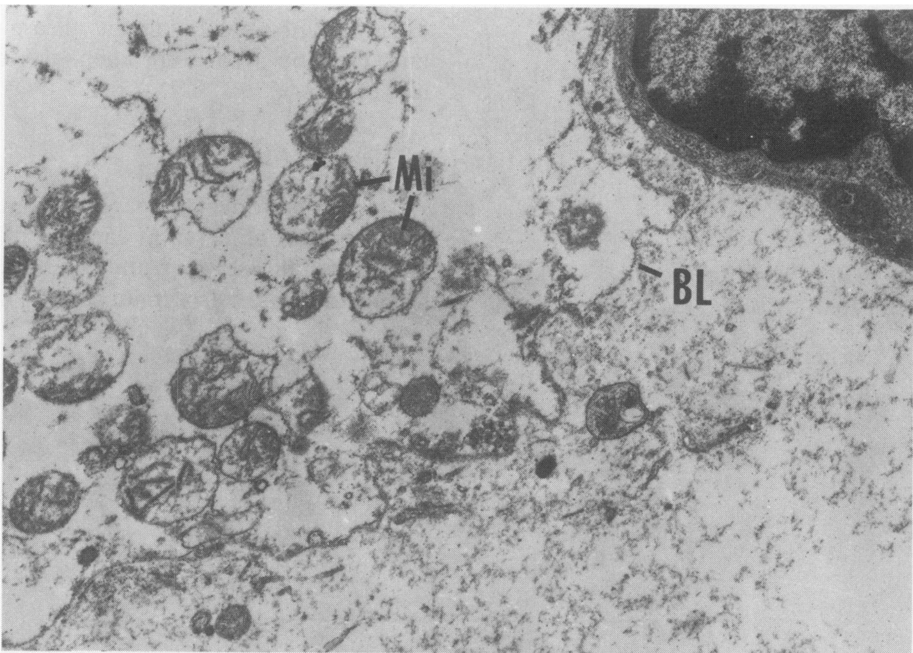

Fig. 6. Electron micrograph of skeletal muscle 24 h after envenomation. Peripheral area of a necrotic cell. The basal lamina (BL) is present, but the plasma membrane has been lost. A group of swollen mitochondria (Mi) are present, some of which have only one membrane. $\times$ 9000.

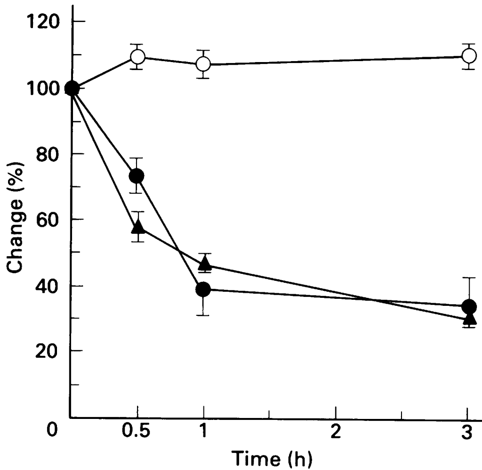

Fig. 7. Changes in the wet weight, and in creatine and CK contents of gastrocnemius muscle at different time intervals after *im* injection of *M. nigrocinctus* venom (30 μg). Results are expressed as percentages, taking as 100% the values of non-injected contralateral gastrocnemius. (○) wet weight; (●) creatine; (▲) CK. Results are presented as mean ± SEM ($n=4$).

increase in calcium levels was detected 1 and 3 h after venom injection (Table 1). Due to the presence of digitonin in the Arsenazo III-containing medium, this increase in calcium mainly reflects cytoplasmic calcium levels (Murphy *et al.* 1980), since digitonin selectively releases cytoplasmic calcium but has little effect on other calcium pools.

Myoglobin in urine and plasma

Mice injected with *M. nigrocinctus* venom had reddish urine and plasma. In order to differentiate between haemoglobin and myoglobin, samples were analysed electrophoretically using a system based on different mobilities. A large amount of myoglobin was present in both plasma and urine, 1 and 3 h after injection. Haemoglobin was not detected in urine and only a little was present in plasma 1 h after envenomation. Thus, myoglobin is the protein responsible for coloration of plasma and urine, corroborating the powerful myotoxicity of this venom.

Myotoxic activity in vitro

M. nigrocinctus venom induced membrane damage in guinea pig extensor digitorum longus muscle *in vitro*, as shown by an increase in efflux of CK and creatine. Their rate of efflux was similar (Fig. 8), suggesting that the functional 'pores' induced by the venom in the membrane were large enough to allow the escape of a macromolecule such as CK. There appeared to be a latent period between venom addition and membrane damage, since no significant release of CK and creatine was seen after 30 min of incubation.

Changes in LDH isozymes

A complex pattern of increase in plasma LDH isozymes was observed as early as 1 h after envenomation and by 3 and 6 h there were further increases. Isozymes LDH-3, LDH-4,

Table 1. Calcium level changes in muscle injected with *M. nigrocinctus* venom

Treatment	Abs $_{675-685}$ in Arsenazo III containing medium*
Saline solution 1 h	0.15 ± 0.02
Saline solution 3 h	0.16 ± 0.02
Venom 1 h	0.23 ± 0.03†
Venom 3 h	0.28 ± 0.02†

* Results are presented as mean ± SEM ($n=4$)

† The values in venom-injected mice were significantly higher ($P<0.01$) than in saline-injected mice.

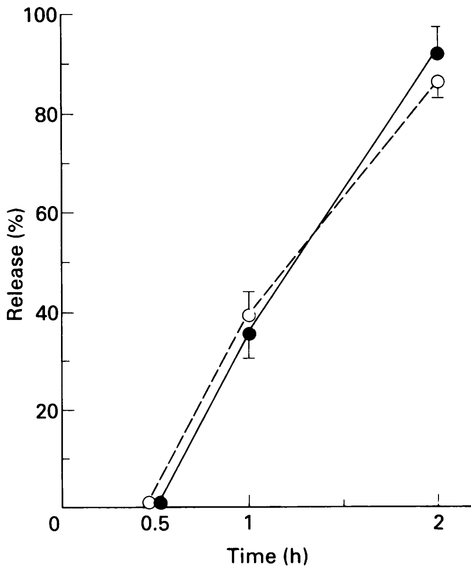

Fig. 8. Release of creatine and CK from guinea pig extensor digitorum longus muscle incubated *in vitro* with *M. nigrocinctus* venom (50 μg/ml) for different time intervals. Results are expressed as percentages, taking as 100% the release of these molecules from muscle incubated for 2 h with a physiological solution containing 1% Triton X-100. (○), CK; (●), creatine. Results are presented as mean ± SEM (n = from 6 to 8).

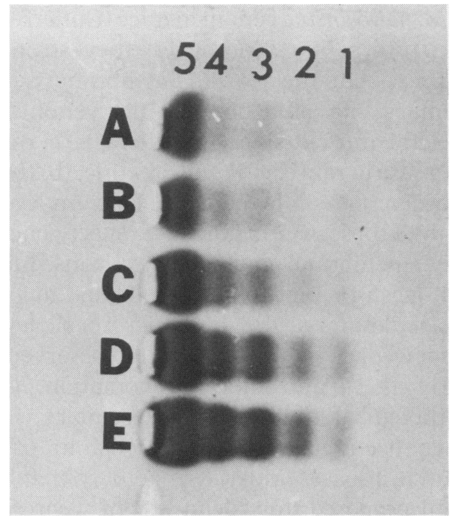

Fig. 9. Electrophoretic pattern of plasma lactate dehydrogenase (LDH) isozymes obtained from mice injected with *M. nigrocinctus* venom. Isozymes LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5 are indicated by 1, 2, 3, 4 and 5, respectively. A and B, samples from control mice; C, D and E, samples obtained at different time intervals after injection of venom; C, 1 h; D, 3 h; E, 6 h. Notice the prominent increase in bands 3, 4 and 5. The anode is to the left.

and LDH-5 showed the largest increment, whereas fractions LDH-1 and LDH-2 increased only slightly (Fig. 9).

Direct haemolysis

M. nigrocinctus venom induced moderate haemolysis of mouse erythrocytes. The haemolysis was more drastic when calcium was present in the incubation media, and only very weak in calcium-free saline solution (Table 2).

Discussion

Our results confirm previous findings of drastic myotoxic activity after im injections

Table 2. Direct haemolytic effect induced by *M. nigrocinctus* venom on mouse erythrocytes

	Haemolysis*	
Venom (μg)	No Ca^{2+}	2 mM Ca^{2+}
10	0	2.7 ± 0.8
100	0	8.5 ± 2.7
500	0	11.7 ± 1.0
1000	0	34.3 ± 4.8

* Results are presented as percentage of haemolysis (mean ± SEM; n = 6)

of *M. nigrocinctus* venom in mice (Gutiérrez *et al.* 1980, 1983). Moreover, they strongly suggest that the plasma membrane is the primary site of action of this venom on skeletal muscle since: (a) there is a rapid decrease in muscle CK and creatine; (b) there is an increase in cytoplasmic calcium levels; both of these events probably reflect generalized membrane damage with consequent changes in permeability to ions and macromolecules; (c) at the light microscopic level, focal wedge-shaped lesions were observed as early as 15 min after envenomation. This pathological finding closely resembles what have been called 'delta lesions' in other muscle diseases (Mokri & Engel 1975). It has been proposed that 'delta lesions' represent focal areas that degenerate after the plasma membrane has been disrupted; (d) ultrastructurally, the earliest morphological alterations were observed in the plasma membrane which was focally disrupted in many places and totally absent in others.

The release of molecules with different molecular weights such as CK and creatine occurred at the same rate indicating the formation of large 'pores' or 'lesions' in the plasma membrane. Thelestam & Mollby (1979) developed a classification of cytolytic toxins based on the size of the plasma membrane lesions which they caused, according to the release of molecules of different weights. In our case, lesions were large enough to allow the escape of a macromolecule such as CK. Thus, the venom does not exert a selective effect on ion permeability but drastically damages the membrane in a less specific way.

There was a difference in the time course of sarcolemmal damage between experiments performed *in vitro* and *in vivo*; in the latter the membrane damage was more rapid. This may be due to the fact that in the *in vivo* experiments the venom was injected intramuscularly, thereby reaching muscle fibres faster. *In vitro* a delay in the onset of sarcolemmal damage was observed, probably because the venom had to penetrate the muscle mass from the outside thus delaying its binding to muscle membranes. Alternatively, the differences may be due to variations in the susceptibility of these two species to the myotoxic components of the venom.

There are similarities between the pathogenesis of myonecrosis induced by *M. nigrocinctus* venom and that induced by other elapid venoms and toxins such as those of *Notechis scutatus* and *Oxyuranus scutellatus* (Harris *et al.* 1975; Harris & Maltin 1982). It has been proposed that these venoms affect directly the sarcolemma of muscle fibres and that the phospholipolytic activity of the toxins notexin and taipoxin is the basis for this myotoxicity (Harris & MacDonell 1981; Harris & Maltin 1982). Other components present in elapid venoms which induce myonecrosis are the 'cardiotoxins', non-enzymatic polypeptides that induce membrane leakiness (Duchen *et al.* 1974).

The most important consequence of plasma membrane damage is the impairment in regulation of its permeability to ions and macromolecules. Of prime significance is the calcium influx, following an electrochemical gradient, since an increase in cytosolic calcium levels is considered a key factor in many instances of muscle degeneration. This calcium influx may be responsible for mitochondrial poisoning (Wrogeman & Pena 1976), as well as the activation of endogenous proteases (Duncan 1978) and phospholipases (Trump *et al.* 1982) which cause further membrane damage.

Another consequence of calcium influx would be the observed hypercontraction of myofilaments. This started with the appearance of hypercontracted portions alternating with overstretched myofibrils, followed by a clumping of myofilaments in which the striated pattern disappeared. Due to the rapid sequence of myofibrillar changes we were not able to detect if a given sarcomeric component was affected earlier than others. Probably the sequence of degenerative events is so rapid that all myofibrillar components are rapidly clumped into amorphous masses. However, proteases such as calcium-activated neutral proteinase could cause

degradation of desmin and alpha-actinin before affecting other myofibrillar proteins, as has been described in the case of plasmocid-induced muscle damage (Ishiura *et al.* 1984). Biochemical analysis of envenomated muscle is necessary to confirm this contention.

Experiments with mouse erythrocytes indicate that the membrane-damaging effect of *M. nigrocinctus* venom varies according to cell type. It is more active on skeletal muscle membrane than on erythrocyte membrane. Similar findings have been described for elapid cardiotoxins (Harvey *et al.* 1983) which seem to have higher specificity for excitable cell membranes. The fact that *M. nigrocinctus* venom induces stronger haemolysis in the presence of calcium suggests a synergistic effect, similar to that observed in cobra venoms, between phospholipase A and cardiotoxin or membrane–active polypeptide (Condrea 1979). Preliminary immunological studies indicate that there are cardiotoxin-like molecules in this venom.

Mice envenomated with coral snake venom excrete reddish urine. The present investigation shows that myoglobin and not haemoglobin is responsible for this phenomenon. Thus, the *in vivo* and *in vitro* results correlate well in that haemolysis is not a significant event following envenomations by *M. nigrocinctus*. On the other hand, the presence of myoglobinuria corroborates the severity of the venon-induced myotoxic effect.

The effects of venom injection on plasma LDH isozymes deserve consideration. There was not a prominent elevation of LDH-1, suggesting that neither intravascular haemolysis, nor myocardial damage are significant pathological features of venom damage. There was, however, a drastic increase of isozymes LDH-3, LDH-4, and LDH-5 suggesting that skeletal muscle is not the only tissue affected, and that the pathophysiology of coral snake envenoming is more complex than previously thought. Increase in isozyme LDH-3 could reflect pulmonary damage (Neremberg 1975), an effect that has not been described in coral snake envenoming. At this time it is not known whether or not this effect was caused by direct action of venom components in the lungs. Further studies on the pathology of animals injected with *M. nigrocinctus* venom are required to develop a comprehensive model of this type of envenomation.

Acknowledgements

The authors thank Alfredo Vargas, Javier Núñez and Abel Robles for their valuable technical help, and Dr Libia Herrero for reviewing the manuscript. The collaboration of Dr Charlotte L. Ownby and the secretarial assistance of Mrs Hilda Herrera are gratefully acknowledged. Part of this work was performed in the Electron Microscopy Unit Universidad de Costa Rica. This project was supported by Vicerrectoría de Investigación, Universidad de Costa Rica. Dr Fernando Chaves received partial support from Consejo Nacional de Investigaciones Científicas y Tecnológicas de Costa Rica (CONICIT).

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