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Pathogenesis of Myonecrosis Induced by Crude Venom and a Myotoxin of *Bothrops asper*

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The pathogenesis of skeletal muscle necrosis induced by crude *Bothrops asper* venom and isolated myotoxic phospholipase was studied using light and electron microscopy. White mice were injected intramuscularly with a dose of 2.5 μg/g and tissue samples were taken at 30 min and 1, 3, 6, 12, 24, and 48 hr. Toxin-injected muscle showed localized wedge-shaped lesions ("delta lesions") by 30 min, which included disrupted plasma membranes. At 1 and 3 hr the predominant type of necrotic cell contained clumped myofibrils in which individual myofilaments were indistinguishable. At later time periods there was a relaxation and redistribution of myofilaments resulting in a more homogeneous and hyaline appearance of necrotic cells. Some mitochondria were swollen and had flocculent densities, and most of them were disrupted, having only one membrane and vesiculated cristae. The basal lamina was intact at all time intervals. Phagocytosis of muscle cell debris started at 3 hr and was prominent by 24–48 hr. In crude venom-injected muscle many cells showed pathologic features identical to those observed after myotoxin injection. Crude venom also induced hemorrhage which was evident 30 min after injection, reaching its highest level by 12 hr. At 3, 6, and 12 hr some cells were undergoing different pathologic changes which appeared to be due to ischemia. Although these cells were irreversibly damaged, as indicated by ruptured plasma membrane, their myofibrillar structure was better preserved than that of toxin-affected cells. The Z line was absent, but A, I, H, and M bands were intact. As a result of Z line loss, sarcomeres were disoriented. It is proposed that the myotoxin induces myonecrosis by first altering the integrity of the plasma membrane, thereby increasing the permeability to calcium, other ions, and molecules which leads to death of the cell. Crude venom affects muscle cells in two ways: by direct action of myotoxin(s) and by ischemia due to hemorrhage.

INTRODUCTION

Poisoning by pit vipers (family Crotalidae) induces myonecrosis, hemorrhage, and edema at the site of venom injection (Ownby, 1982). In Central America, *Bothrops asper* is the species responsible for the majority of snakebite cases (Bolaños, 1982). Its venom induces a drastic myotoxic effect which has been described in both experimental animals and humans (Gutiérrez *et al.*, 1980; Arroyo and Gutiérrez, 1981; Bolaños, 1982). Furthermore, it has been concluded that the polyvalent antivenom available is only partially able to neutralize this effect (Gutiérrez *et al.*, 1981).

Recently, a myotoxin from *B. asper* venom was purified to homogeneity (Gutiérrez *et al.*, 1983). It is a basic phospholipase A with a molecular weight of 10,700, it induces myonecrosis in mice, and it is able to hydrolyze muscle phospholipids *in vivo*. This toxin may prove to be a useful tool in understanding the mode by which this venom affects skeletal muscle. In this communication we describe the pathogenesis of myonecrosis induced by this toxin, as well as by crude *B. asper* venom, at the light and electron microscopic levels. Also, the development of hemorrhage in skeletal muscle induced by the crude venom was

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studied in an attempt to correlate changes in microcirculation with the pathogenesis of myonecrosis.

MATERIALS AND METHODS

Venom. Bothrops asper venom was a generous gift from Dr. Luis Cerdas, Instituto Clodomiro Picado, Universidad de Costa Rica. The venom is a pool obtained from more than 50 specimens collected in the Atlantic slopes of Costa Rica.

Toxin. Myotoxin was isolated according to the procedure described by Gutiérrez *et al.* (1984). Homogeneity was shown by disk-polyacrylamide gel electrophoresis (pH 4.3) as previously described (Gutiérrez *et al.*, 1984).

Histological and ultrastructural studies. Groups of four female mice (Charles River, CD-1) weighing 20 ± 2 g were injected intramuscularly (i.m.) dorsolaterally in the thigh with venom (2.5 μg/g), toxin (2.5 μg/g), or physiologic saline solution. At seven time intervals (30 min, 1, 3, 6, 12, 24, and 48 hr) mice were killed by cervical dislocation and a sample of muscle obtained from the ventromedial aspect of the thigh. The tissue was processed for light and electron microscopy as previously described (Ownby *et al.*, 1976). Thick sections were stained with Mallory's trichrome, and thin sections (silver to light gold) were stained with methanolic uranyl acetate and lead citrate, and examined in a Philips EM 200 electron microscope. A total of 68 mice were used.

Quantitation of hemorrhage. Local hemorrhage was quantitated according to the method of Ownby *et al.* (1984) in which the amount of hemoglobin in muscle extracts is measured after i.m. injection of venom (2.5 μg/g) in mice. Hemoglobin content is expressed as corrected hemoglobin (gram percent). This value is obtained by taking into consideration the weight of muscle from which the extract was made.

RESULTS

Myonecrosis Induced by the Toxin

Light microscopy. Samples obtained 30 min after injection contained degenerating as well as necrotic and normal cells. Many cells had focal, wedge-shaped lesions, the bases of which were at the surfaces of the cells with the apexes pointing toward the interiors (Fig. 1a). These lesions closely resemble the "delta lesions" described by Mokri and Engel (1975) in biopsies from Duchenne muscular dystrophy patients. Some cells had several of these lesions (Fig. 1a). At 30 min, 1 hr, and 3 hr, many cells were necrotic, with conspicuous clumps of myofibrils alternating with empty spaces in the cytoplasm (Fig. 1b). In these cells the striated pattern was absent. By 3 hr, but especially at 6, 12, 24, and 48 hr, the appearance of these cells gradually changed as the clumped morphology of the myofibrils disappeared (Fig. 1c). The cytoplasm became more hyaline and homogeneous. Phagocytosis started at 3 hr, but was not prominent until 12, 24, and 48 hr (Fig. 1d). The toxin did not affect blood vessels, nerves, or muscle spindles. No change was observed in muscle obtained from mice injected with physiologic saline solution.

Electron microscopy. Examination of "delta lesions" at 30 min showed that the plasma membrane was focally or, more often, completely disrupted (Fig. 2).

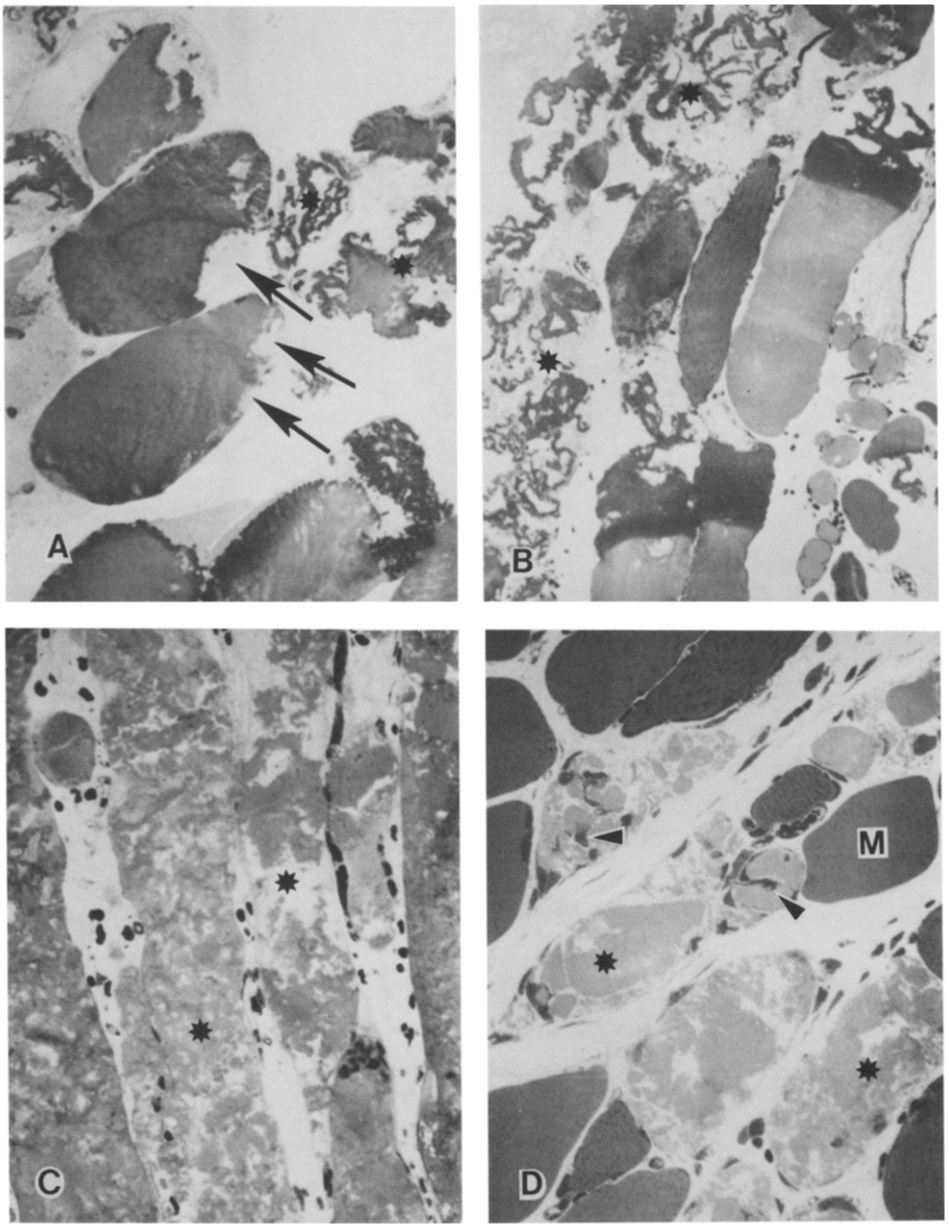

FIG. 1. Light micrographs of skeletal muscle taken at various times after injection of *B. asper* myotoxin. (A) 30 min: "Delta lesions" (arrows) are the earliest changes observed. Other necrotic cells (*) contain clumped myofibrils (×350). (B) 1 hr: Necrotic cells (*) with clumped myofibrils are present (×200). (C) 6 hr: Necrotic cells (*) have a hyaline appearance in which the myofibrillar material has a more homogeneous pattern (×350). (D) 48 hr: Necrotic cells (*) with a hyaline appearance are located between normal muscle cells (M). Phagocytic cells (arrowheads) are observed in the connective tissue and within the basal lamina of necrotic cells (×350).

Beneath these membrane defects the myofibrils were clumped into dense masses, leaving as a consequence many empty spaces in the cytoplasm. Mitochondria were swollen, disrupted, and contained vesiculated cristae (Fig. 2). Alteration of the sarcoplasmic reticulum resulted in many small vesicles randomly located in

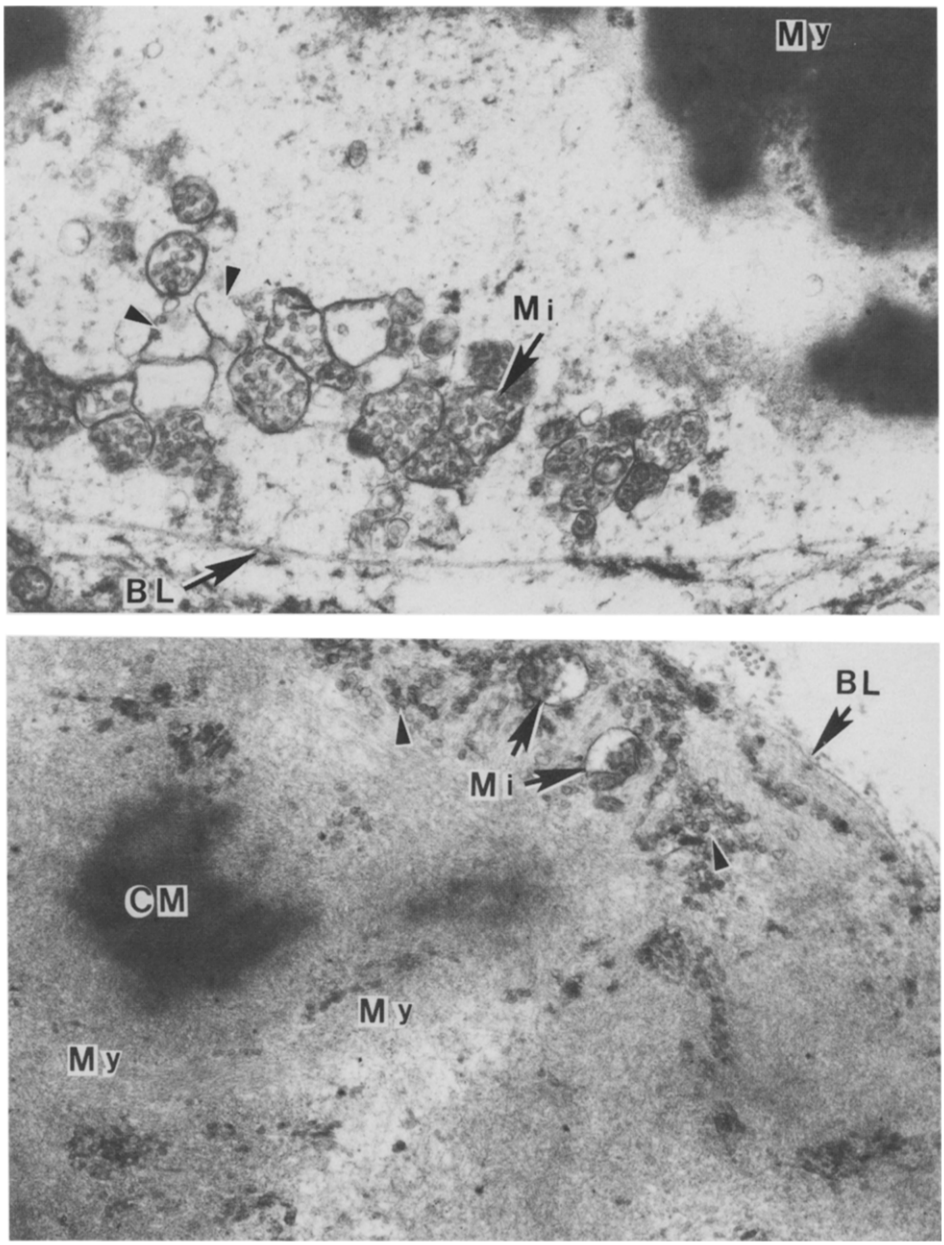

FIG. 2–5. Electron micrographs of skeletal muscle taken at various times after injection of *B. asper* myotoxin.

FIG. 2. (top, 30 min) Portion of a muscle cell with a "delta lesion." Note that the plasma membrane is no longer present, whereas the basal lamina (BL) is intact. Myofilaments (My) are clumped into dense masses. Some mitochondria (Mi) have vesiculated cristae, whereas others are disrupted (arrowheads) (×24,000).

FIG. 3. (bottom, 3 hr) Necrotic cell undergoing a transition from clumped to hyaline appearance. Myofilaments (My) are being dispersed from a clumped mass (CM). The plasma membrane is not present, but the basal lamina (BL) is intact. Many small vesicles are present in the cytoplasm (arrowheads), and mitochondria (Mi) are swollen and disrupted (×12,500).

the affected areas. At later time periods, there were cells in which practically all the organelles were severely affected, with the exception of the basal lamina which was intact (Fig. 3). This was the predominant pattern of necrosis at 30 min, 1 hr, and 3 hr. By 3 hr some cells appeared to be in a transition to a different necrotic stage, in which the cells no longer had a clumped appearance as myofilaments at the edge of the clumps became dispersed (Fig. 3). These myofilaments were devoid of any pattern of organization. Later, at 6, 12, 24, and 48 hr, the myofibrillar material in all necrotic cells was loosely packed and disorganized. By 6, 12, 24, and 48 hr, muscle cells showed further degradation of the rest of the organelles, and the plasma membrane was almost completely absent (Fig. 4). Normal sarcoplasmic reticulum and T tubules were rare, but there were many small, round vesicles randomly dispersed in the cytoplasm. Phagocytosis was observed at 3 hr, but it was more pronounced by 12, 24, and 48 hr. Phagocytic cells were observed inside the basal lamina, and mitochondria, vesicles, and myofilaments were present inside phagocytic vacuoles (Fig. 4). Mitochondrial alterations were prominent at all time intervals. Many mitochondria were swollen and had dense intracristal spaces, others contained flocculent densities, and most of them had vesiculated cristae and only one membrane which was frequently broken (Fig. 5). Capillaries and connective tissue cells were not affected by the toxin. The ultrastructure of muscle injected with physiologic saline solution was normal.

Myonecrosis Induced by Crude Venom

Light microscopy. Two different patterns of muscle cell necrosis were observed after crude venom injection. One of them closely resembles the action of the toxin. In this pattern, early changes were observed by 30 min, with the presence of "delta lesions." By 1 and 3 hr these cells developed the characteristic clumping of myofibrils (Fig. 6a). On the other hand, by 3, 6, and 12 hr some cells were going through a different series of pathologic changes. Their myofibrils were neither clumped nor did they have a hyaline appearance. Instead, their striated appearance was partially lost, but the myofibrillar architecture seemed to be better preserved (Fig. 6b). At later stages, necrotic cells appeared hyaline with an amorphous homogeneous cytoplasm. After the third hour, there was a phagocytic infiltration which peaked by 24–48 hr. Besides its action on muscle cells, *B. asper* venom induced prominent hemorrhage, as many erythrocytes were observed in the interstitial connective tissue.

Electron microscopy. There were many necrotic cells that had almost identical characteristics to toxin-affected muscle cells. By 30 min many cells showed focal disruptions in the continuity of the plasma membrane (Fig. 7). At 30 min, and especially at 1 and 3 hr, the predominant necrotic cell type had the following characteristics: disrupted plasma membrane, intact basal lamina, and myofilaments that were coalesced into dense, clumped masses in which it was impossible to differentiate individual myofilaments (Fig. 8). Some mitochondria were swollen, others had flocculent densities, and still other mitochondria had lost their integrity, as was described for toxin-injected muscle (Fig. 5). Sarcoplasmic reticulum disruption resulted in the formation of many small, rounded vesicles, whereas T tubules could not be observed. Nuclei showed clumping of chromatin and separation of chromatin from the nuclear envelope which was often discontinuous (Fig. 8). By 6, 12, 24, and 48 hr these necrotic cells had a different

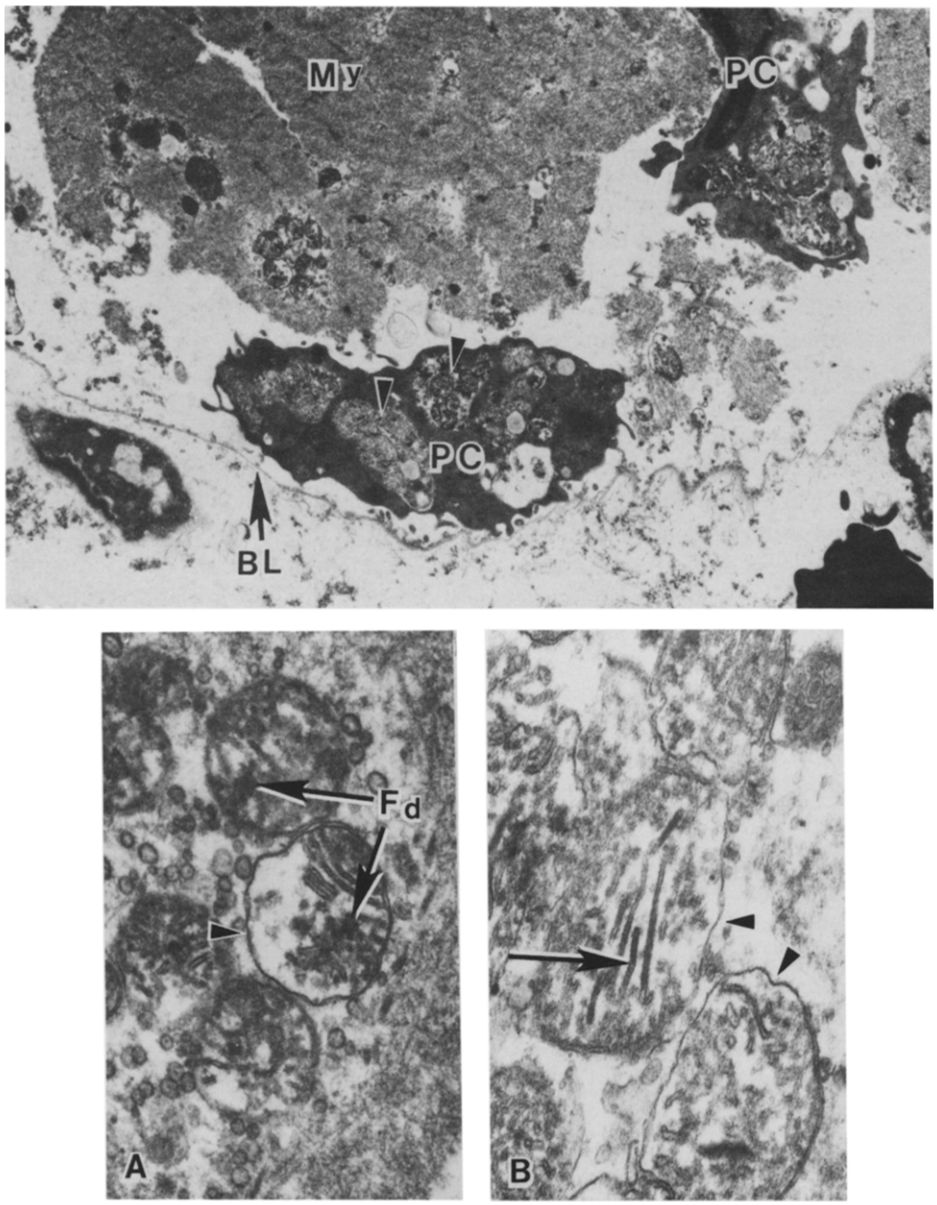

FIG. 4. (top, 24 hr) Necrotic muscle cell invaded by phagocytic cells (PC). Note the presence of mitochondria inside phagocytic cells (arrowheads). Myofibrillar material (My) appears as an amorphous mass. The plasma membrane is not present, but the basal lamina (BL) is intact (×5000).

FIG. 5. Mitochondrial changes. (A) 24 hr after toxin injection. Swollen mitochondria containing flocculent densities (Fd) are present. There is only one membrane in some areas (arrowhead) (×29,000). (B) 6 hr after toxin injection. Mitochondria are swollen and have only one membrane (arrowheads) as well as dense intracristal spaces (arrow) (×31,500).

morphology in which there was a gradual relaxation and redistribution of the clumped myofibrils so that individual myofilaments and groups of myofilaments were observed in the cytoplasm. Otherwise, the rest of the organelles had the same alterations described above. Significantly, the basal lamina remained intact

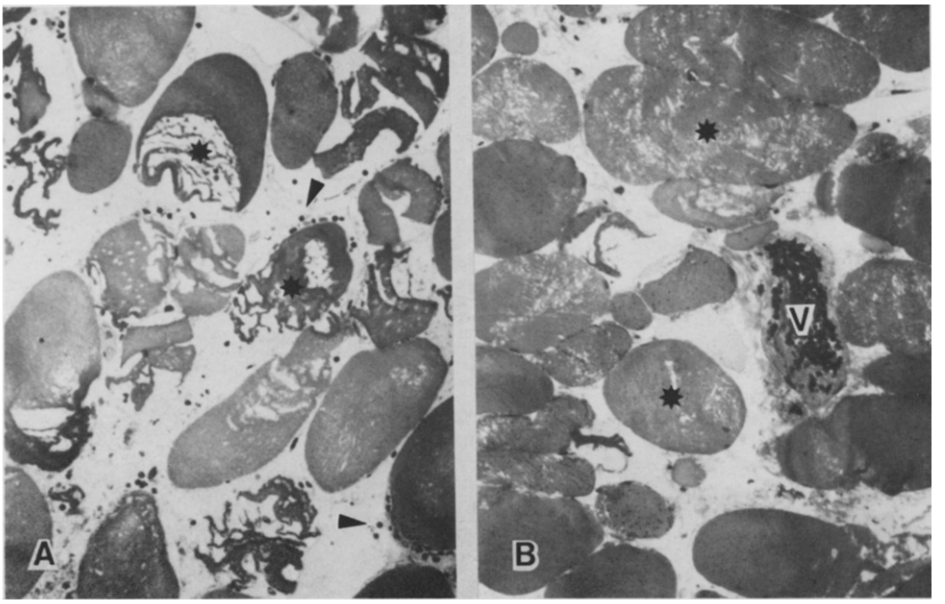

FIG. 6. Light micrographs of skeletal muscle taken at various times after injection of *B. asper* crude venom. (A) 1 hr, necrotic cells (*) contain clumped myofibrils. Erythrocytes (arrowheads) are present in the endomysium (×250). (B) 6 hr, necrotic cells (*) in which the myofibrillar structure is partially preserved. This pattern of necrosis was observed in some areas at 3, 6, and 12 hr. A congested blood vessel (V) has many erythrocytes in its lumen (×250).

in necrotic cells. Erythrocytes were observed in the interstitial connective tissue as well as inside necrotic cells.

At 3 hr, and especially at 6 and 12 hr, many cells seemed to be going through a different series of pathologic changes. These cells were evidently altered, but their myofibrillar structure was better preserved than in other necrotic cells. In these cells the sarcomeric organization was preserved, with the exception of the Z line which was selectively absent. Otherwise, A, I, H, and M bands could be identified (Fig. 9a). As a consequence of Z line loss, the sarcomeres were disoriented, with the consequent lack of striated pattern. Thus, the mechanical integration of myofibrils was obviously impaired in these cells which were irreversibly damaged, since their plasma membrane was disrupted in many areas. Their mitochrondria were swollen and had vesicles in their interior, whereas others showed dense intracristal spaces. Sarcoplasmic reticulum membranes were broken into small vesicles, but the basal lamina was intact (Fig. 9b). This pattern of necrosis was absent by 24 and 48 hr, indicating that these cells progressed towards the hyaline, homogeneous morphology described for the action of the toxin at later time periods (Fig. 4).

Quantitation of hemorrhage. Hemorrhage developed very rapidly after *B. asper* venom injection. By 30 min there was a conspicuous increase in the hemoglobin content of the muscle. Local hemorrhage increased up to 12 hr at which time this activity reached its maximum level (Fig. 10).

DISCUSSION

A myotoxic phospholipase A has been isolated from *B. asper* venom. When injected i.m. into mice it induces necrosis of skeletal muscle. Both toxin and

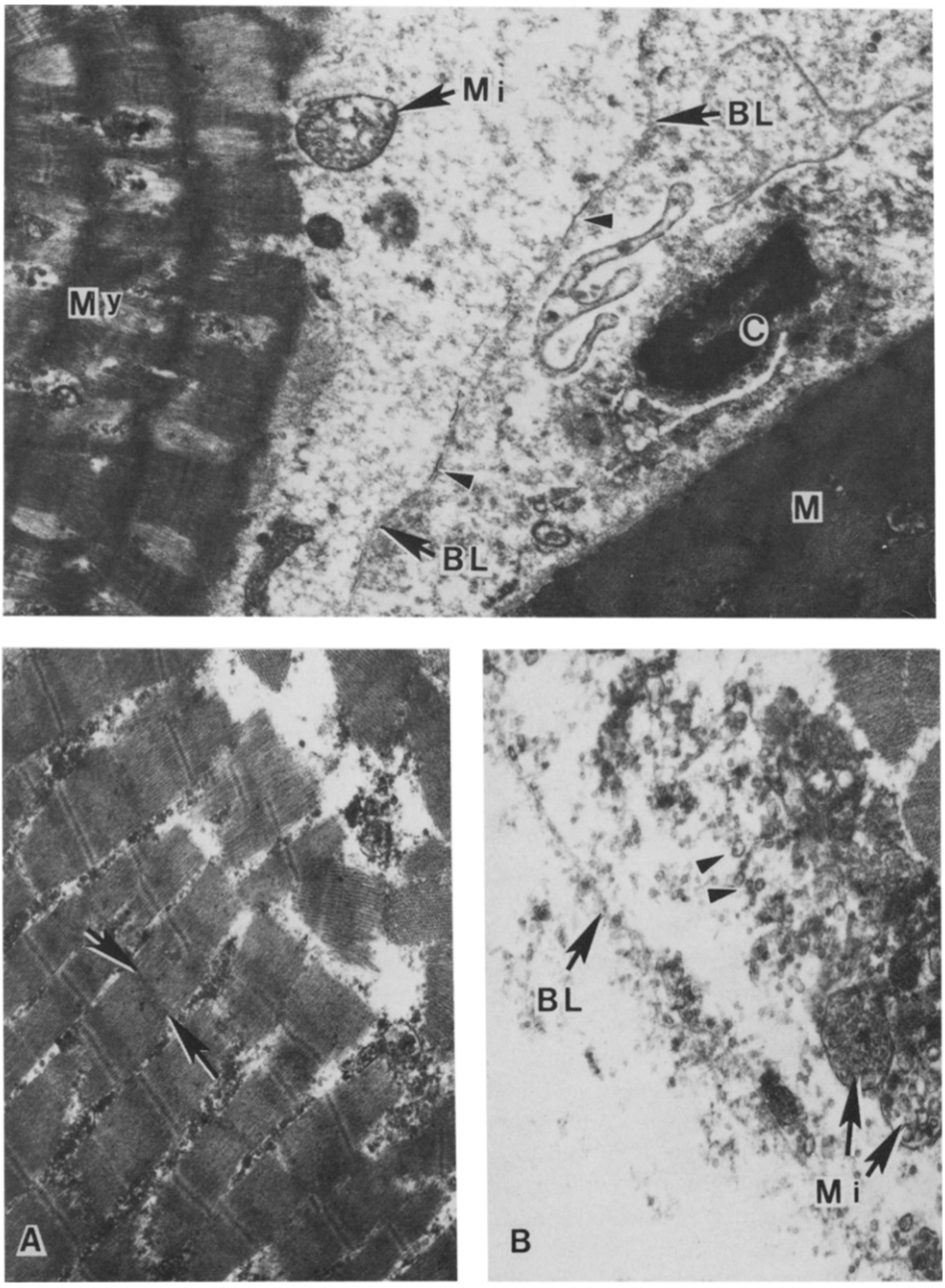

FIG. 7–9. Electron micrographs of skeletal muscle taken at various times after injection of crude *B. asper* venom.

FIG. 7. (top, 30 min) Portions of two muscle cells, one of which is normal (M) whereas the other (left side of micrograph) is undergoing early pathologic changes. In the abnormal cell, the plasma membrane is present only in small patches (arrowheads), whereas the basal lamina (BL) is intact. Myofibrils (My) are disoriented and mitochondria (Mi) are swollen. Note the disrupted capillary (C) in the endomysium (×14,000).

FIG. 9. (bottom, 6 hr) (A) Portion of a muscle cell to show alteration of sarcomere structure. Z line is absent (arrows), whereas A, I, H, and M bands are present. Some sarcomeres seem to be dispersed in the cytoplasm which contains many vesicles (×15,500). (B) Mitochondria (Mi) are swollen, contain vesiculated cristae, and some have only one membrane. Many vesicles (arrowheads) are randomly dispersed in the cytoplasm, and the plasma membrane is not present; the basal lamina (BL) is intact (×19,500).

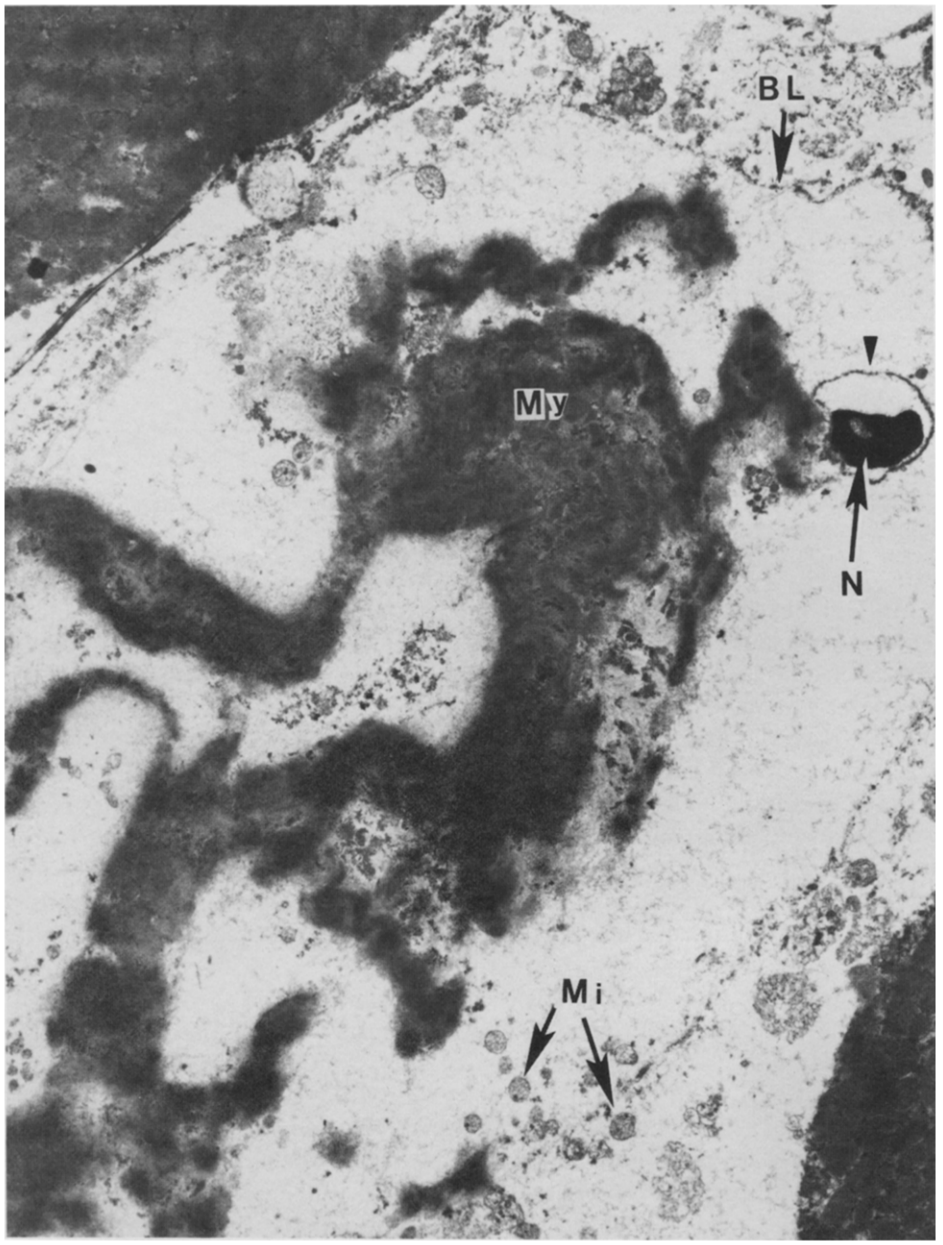

FIG. 8. (3 hr) Electron micrograph of skeletal muscle taken at 3 hr after injection of crude *B. asper* venom. Typical necrotic cell with clumped myofibrils (My). Mitochondria (Mi) are swollen and contain flocculent densities; plasma membrane is absent in many areas, but the basal lamina (BL) is intact. Note that the nucleus (N) is pycnotic and the dense chromatin is separated from the nuclear envelope (arrowhead) (×4500).

venom act very fast, as many cells are irreversibly damaged within 30 min after injection.

In the pathogenesis of myonecrosis, the earliest changes in affected fibers seem to be related to the plasma membrane. Thirty minutes after injection of both toxin and venom there were many wedge-shaped lesions in the cells. Such lesions have been called "delta lesions" and represent areas of cell degeneration located beneath portions of disrupted or discontinuous plasma membrane (Mokri and Engel,

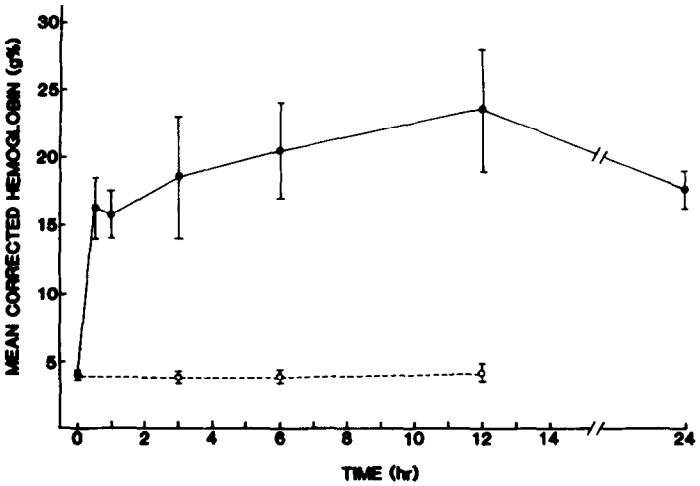

FIG. 10. Development of hemorrhage in muscular tissue after i.m. injection of *B. asper* venom (2.5 μg/g). Tissue was homogenized and the hemoglobin content estimated according to the procedure of Ownby *et al.* (1984). Venom (●); physiologic saline solution (○).

1975). Our observations at the ultrastructural level indicate an almost total absence of plasma membrane in these areas. Similar lesions have been observed in biopsies from Duchenne muscular dystrophy patients (Mokri and Engel, 1975), as well as after injections of membrane-active substances such as lysolecithin, mellitin, deoxycholate, and Triton X-100 (Pestronk *et al.*, 1982). In all of these cases the plasma membrane was affected both morphologically and functionally. A previous study showed that *B. asper* myotoxin induces a calcium influx into skeletal muscle cells and a rapid rise in plasma creatine kinase levels during the first hours after injection (Gutiérrez *et al.*, 1984). Also, the toxin is able to hydrolyze muscle phospholipids *in vivo*. These events are probably indications of membrane disruption and support the view that the plasma membrane is the primary site of action of this toxin.

It is interesting that the pathologic changes caused by *B. asper* myotoxin resemble those induced after inoculations of several venoms and toxins such as notexin (Harris *et al.*, 1975), taipoxin (Harris and Maltin, 1982), crotoxin (Hawgood, 1982), a cardiotoxin (Duchen *et al.*, 1974), tarantula venom (Ownby and Odell, 1983), and phospholipase C from *Clostridium perfringens* (Strunk *et al.*, 1967). Similar observations have been made in biopsies from Duchenne muscular dystrophy patients (Mokri and Engel, 1975; Cullen and Fulthorpe, 1975). In all these cases it has been postulated that the primary basic change consists in lesions of the plasma membrane, with a subsequent impairment of its ability to regulate the permeability to ions and molecules.

The most significant consequence of such membrane disruption is the influx of calcium following an electrochemical gradient that is normally maintained across the cell membrane (Trump *et al.*, 1981; Carafoli, 1982). Calcium influx has been observed to occur after *B. asper* myotoxin injection (Gutiérrez *et al.*, 1984). An increase in cytosolic calcium levels has been considered a key factor in the process of cell injury in many pathologic conditions (Farber, 1982; Trump *et al.*, 1981). In skeletal muscle cells such a rise in calcium levels results in (a) hypercontraction and clumping of myofilaments; (b) mitochondrial overload which results in swelling and the formation of flocculent densities (Publicover *et al.*, 1977; Wrogemann and Pena, 1976); (c) activation of calcium-dependent proteases

(Duncan, 1978), such as the calcium-activated neutral proteinase isolated from skeletal muscle (Ishiura, 1981); and (d) activation of calcium-dependent phospholipases, responsible for further degradation (not only of plasma membrane, but also of sarcoplasmic reticulum and mitochondrial membranes (Trump *et al.*, 1981).

As a consequence of venom and toxin injections, necrotic cells show a prominent clumping of myofilaments in dense masses. Later on, these clumps began to relax and there was a homogenization or redistribution of the myofibrillar material in the cellular space. This resulted in the hyaline, homogeneous appearance of cells which predominated at later time periods. A similar observation has been made in biopsies from Duchenne muscular dystrophy patients where a process of gradual homogenization of the clumped contractile material was described (Cullen and Fulthorpe, 1975). These two morphologic patterns seem to be stages of the same process of cell injury. This transition is demonstrated by the fact that at 3 hrs there are cells in which the dense clumps are undergoing dissolution.

With regard to myonecrosis induced by crude venom, many cells had pathological features identical to those described for toxin-affected cells. Nevertheless, there were also groups of cells that presented different morphologic characteristics. In these cells, there was not a dramatic clumping of myofilaments, and the myofibril structure was better preserved. Several lines of evidence indicate that these cells may be affected by ischemia. Morphologically they resemble the skeletal muscle cells described by Karpati *et al.* (1974) in experimentally-induced ischemia, which are characterized by disrupted plasma membrane, vesiculated sarcoplasmic reticulum, and pycnotic nuclei. Moreover, these authors also observed disappearance of Z line, although myofilaments were still bundled in myofibrils. All of these morphologic characteristics were observed in some cells 3, 6, and 12 hr after injection of *B. asper* venom. In venom-affected cells, mitochondria were swollen and some of them had intracristal plates similar to the ones observed by Karpati *et al.* (1974) in ischemic muscle. Hanzlíková and Schiaffino (1977) described giant mitochondria in ischemic muscle. These were not observed in our experiments. With regard to Z line loss, it is significant that a calcium-activated neutral proteinase isolated from skeletal muscle releases several proteins upon incubation with myofibrils, with the disappearance of Z line (Ishiura, 1981). Furthermore, *B. asper* venom induces local hemorrhage which reaches significant levels as early as 30 min after injection. Thus, this compromise of local microcirculation may impair the adequate blood supply to the tissue, inducing ischemia in some areas. It has been proposed that viriditoxin, a hemorrhagic toxin isolated from the venom of *Crotalus viridis viridis,* induces an ischemic condition in skeletal muscle which in turn is responsible for the muscle necrosis observed after inoculation of this toxin (Gleason *et al.*, 1983). Thus, the action of *B. asper* venom in muscle is a complex one, since there is direct damage to cells by the myotoxin, as well as indirect effects due to ischemia, perhaps induced by hemorrhagic toxins.

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