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Inflammatory infiltrate in skeletal muscle injected with *Bothrops asper* venom

José María Gutiérrez, Fernando Chaves and Luis Cerdas.
Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica. San José, Costa Rica.

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Abstract: The time-course and composition of inflammatory infiltrate in mouse gastrocnemius injected with *Bothrops asper* venom was studied. The venom induced myonecrosis, and a prominent decrease in muscle levels of creatine kinase (CK) as early as 3 hr after envenomation. Inflammatory infiltrate was scarse by 6 hr. but increased markedly at 24, 48 and 72 hr. Samples of infiltrate obtained at 6 and 24 hr contained polymorphonuclear leucocytes as the predominant cell type, whereas at 48 hr and 72 hr the relative number of macrophages increased. Inflammatory cells were located within necrotic muscle cells, as well as in the interstitial space, but there were some necrotic areas devoid of inflammatory cells even one week after envenomation. When correlating the presence of inflammatory cells with degradation of myofibrillar proteins, it was observed that at 6 hr there was little muscle protein degradation. By 48 hr a decrease in "non collagen" proteins was observed, together with a reduction in some myofibrillar components, as judged by electrophoresis. Proteolytic enzymes of inflammatory cells may play an important role in myofibrillar protein degradation after myonecrosis induced by *B. asper* venom.

A variety of snake venoms induce skeletal muscle necrosis (Tu, 1977; Ownby, 1982). In the case of crotaline venoms, severe vascular alterations such as hemorrhage and thrombosis occur in envenomated muscle (Homma and Tu, 1971; Gutiérrez and Chaves, 1980). Simultaneously, a complex inflammatory response takes place. There have been few studies on the time-course of inflammation after venom injection, particularly in regard to the cellular infiltrate.

Bothrops asper venom induces severe local effects which often result in sequelae such as tissue loss (Gutiérrez *et al.*, 1982; Bolaños, 1982). The pathogenesis of myonecrosis induced by this venom has been studied (Gutiérrez *et al.*, 1984), and it has been observed that an abundant inflammatory infiltrate is present in necrotic muscle several hours after envenomation. The present work was designed to study the time-course of inflammatory infiltrate after myonecrosis induced by *B. asper* venom. Particular attention was paid to the identification of cellular types in the infiltrate and to the relationship between infiltrate and degradation of myofibrillar proteins.

MATERIAL AND METHODS

Venom: *Bothrops asper* venom was obtained from more that 50 adult specimens collected in the Atlantic region of Costa Rica. Once obtained, it was frozen, lyophilized, and stored at -70°C.

Determination of creatine kinase (CK) and non-collagen protein: Groups of four mice (20-22 g) were injected i.m. in the right gastrocnemius with 100 μg of venom (in 0.1 ml of saline solution). At different time intervals (3 hr, 6 hr, 24 hr, 48 hr and 72 hr) animals were killed by cervical dislocation and both gastrocnemius were obtained. Then, the muscle was homogenized in 5.0 ml of phosphate-buffered saline (pH 7.2) containing 0.1% Triton X−100 and centrifuged at 10,000 Xg for 15 min. The supernatant was collected and assayed for creatine kinase (CK) using the Sigma kit No. 520. CK content of envenomated muscle was

expressed in percentage, taking as 100% the CK content of the contralateral gastrocnemius. In samples obtained 6 and 48 hr after envenomation, the pellet was resuspended in 5.0 ml of 0.5 M NaOH and allowed to stand overnight at room temperature. The solution was then centrifuged and the protein content ("non-collagen protein") of the supernatant was determined according to Lowry *et al.* (1951). Non-collagen protein of envenomated gastrocnemius was expressed in percentage, taking as 100% the values of the contralateral muscle.

Electrophoresis of muscle proteins: The pellet resultant from centrifugation of muscle homogenate was resuspended in 250 µl of distilled water and an aliquot was analyzed by slab SDS-polyacrylamide gel electrophoresis (SDS–PAGE), using 12% acrylamide (Laemmli, 1970). SDS-PAGE was performed in samples obtained 6 hr and 48 hr after envenomation.

Studies on the inflammatory infiltrate: The technique of Maskrey *et al.* (1977) was used with some modifications. Groups of four mice (20-22 g) were injected i.m. in the right gastrocnemius with 100 µg of venom. At different time intervals (6 hr, 24 hr, 48 hr and 72 hr) mice were killed and envenomated muscle was removed and chopped with scissors in 2.0 ml of phosphate-buffered saline. The tissue suspension was then incubated, with continuous agitation, for 30 min at 37 C. The suspension was filtered through bolting silk and inflammatory cells were counted in a hemocytometer. Just before counting, the cell suspension was treated with a solution (3% acetic acid) that lyses erythrocytes while leaving leucocytes and macrophages intact; this was done in order to count only inflammatory cells and not erythrocytes present in the tissue due to hemorrhage. Then, the suspension was centrifuged in a cytocentrifuge and smears were prepared and stained with Wright in order to identify the inflammatory cells. Cells were classified as macrophages, polymorphonuclear leucocytes and lymphocytes.

Histology and ultrastructure: Groups of four mice (20-22 g) were injected as described above. At different time intervals, animals were killed and pieces of envenomated muscle were obtained. Samples for histology were processed routinely. Other samples were processed for electron microscopy and embedded in Spurr resin (Arroyo and Gutiérrez, 1981). Thin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HU-12A electron microscope of the Electron Microscopy Unit, Universidad de Costa Rica. Control mice were injected with 0.1 ml of phosphate-buffer saline solution (pH 7.2).

RESULTS

Quantitation of inflammatory infiltrate: Control gastrocnemius muscle contained 85,000 ± 7,000 leucocytes. There was a significant increase ($P < 0.005$) in muscle obtained 6 hr after envenomation (167,500 ± 12,500). By 24 hr, a further increase was observed, reaching values of 810,000 ± 125,000 inflammatory cells per gastrocnemius. The most prominent elevation in inflammatory infiltrate occured at 48 hr (2,400,000 ± 760,000); by 72 hr the infiltrate increased only slightly when compared to values at 48 hr (Fig. 1).

Cell types present in infiltrate: Examination of inflammatory cells by light microscopy allowed us to classify them in three groups: Macrophages, lymphocytes and polymorphonuclear leucocytes. Among the latter, the vast majority of cells were neutrophils. In control muscle, 48 ± 3% of the cells were macrophages, 44 ± 5% were polymorphonuclear leucocytes and 9 ± 1% were lymphocytes. Infiltrate from samples obtained 6 and 24 hr after envenomation presented a high proportion of polymorphonuclear leucocytes (Table 1). By 48 hr there was a change in this pattern; the relative number of macrophages increased and that of polymorphonuclear leucocytes decreased. This trend remained in samples obtained at 72 hr, when macrophages were the predominant cell type in the infiltrate. The percentage of lymphocytes in inflammatory infiltrate did not change, their values ranging from 2 ± 3% to 7 ± 1.3% at all time periods (Table 1).

Determination of creatine kinase in muscle: CK content of injected gastrocnemius decreased to values of 35 ± 3% 3 hr after envenomation. Thereafter there was no significant change in CK content (32 ± 6% at 6 hr; 31 ± 6% 24 hr; and 30 ± 5% at 48 hr).

TABLE 1

Distribution of inflammatory cell types in muscle injected with Bothrops asper *venom* *

Cell types	Control muscle	Envenomated muscle (6 hr)	Envenomated muscle (24 hr)	Envenomated muscle (48 hr)	Envenomated muscle (72 hr)
Macrophage	48 ± 3%	23 ± 3%	14 ± 1%	33 ± 8%	53 ± 2%
Polymorphonuclear Leucocyte	45 ± 2%	72 ± 5%	84 ± 2%	60 ± 9%	40 ± 3%
Lymphocyte	7 ± 1%	5 ± 1%	2 ± 0.3%	7 ± 1%	7 ± 1%

* Results are expressed in relative terms (percentage; mean ± SEM; n = 4), taking as 100% the total number of inflammatory cells present in the sample.

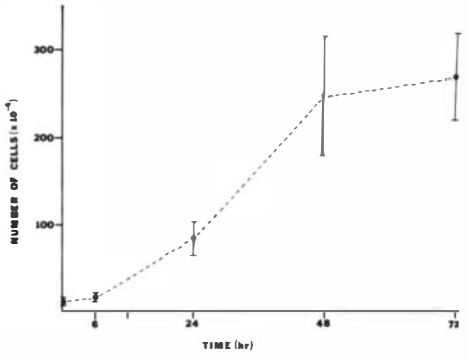

Fig. 1. Time-course of inflammatory infiltrate in mouse gastrocnemius injected with 100 μg of *Bothrops asper* venom. Each value represents the mean ± SEM (n = 4).

Change in myofibrillar proteins: Degradation of myofibrillar proteins was almost negligible in muscle samples obtained 6 hr after venom injection. At this time, non-collagen proteins had decreased to 95 ± 2% when compared to control gastrocnemius. Electrophoretic analysis of muscle proteins indicated that none of the major myofibrillar proteins had been extensively degraded (Fig. 2). There was evidence of protein degradation in muscle obtained 48 hr after envenomation. In these samples, non-collagen protein decreased to a value of 79 ± 6% when compared to control gastrocnemius. Moreover, there was a decrease in the intensity of several bands in SDS–PAGE of muscle proteins (Fig. 2). Both of these observations indicate that a process of myofibrillar protein degradation was taking place.

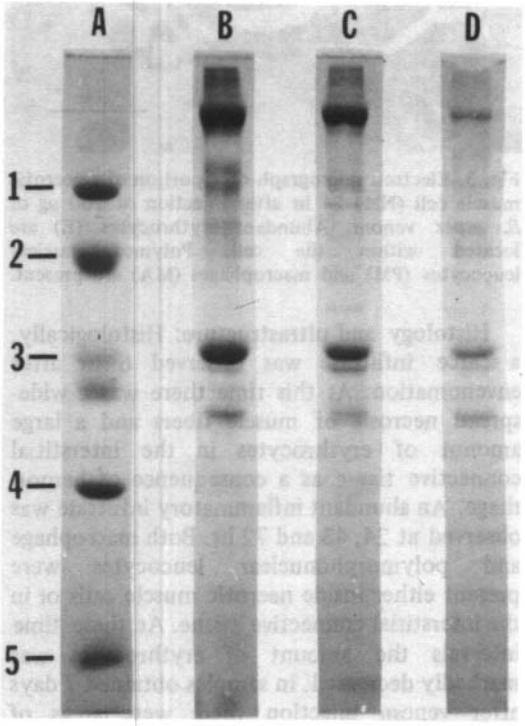

Fig. 2. SDS–PAGE of myofibrillar proteins obtained from mouse gastrocnemius. Lane A: Molecular weight markers; (1) phosphorylase b (94K); (2) albumin (67 K); (3) ovalbumin (43 K); (4) carbonic anhydrase (30 K); and (5) Trypsin inhibitor (20 K). Lane B: Proteins of control muscle. Lane C: Proteins of muscle obtained 6 hr after injection of 100 μg of *B. asper* venom. Lane D: Proteins of muscle obtained 48 hr after venom injection.

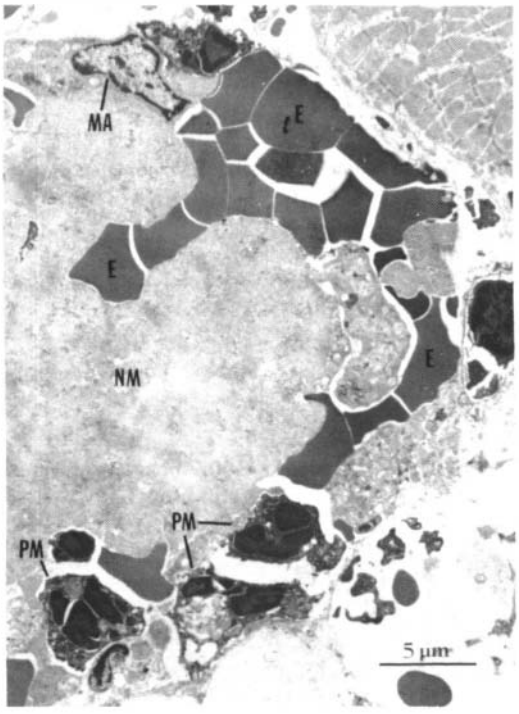

Fig. 3. Electron micrograph of a portion of a necrotic muscle cell (NM) 24 hr after injection of 100 µg of *B. asper* venom. Abundant erythrocytes (E) are located within the cell. Polymorphonuclear leucocytes (PM) and macrophages (MA) are present.

Histology and ultrastructure: Histologically, a scarce infiltrate was observed 6 hr after envenomation. At this time there was a widespread necrosis of muscle fibers and a large amount of erythrocytes in the interstitial connective tissue as a consequence of hemorrhage. An abundant inflammatory infiltrate was observed at 24, 48 and 72 hr. Both macrophage and polymorphonuclear leucocytes were present either inside necrotic muscle cells or in the interstitial connective tissue. At these time intervals the amount of erythrocytes was markedly decreased. In samples obtained 7 days after venom injection there were areas of muscle regeneration as well as areas of granulation tissue; however, there were some portions in which necrotic muscle cells had not been cleared out. In these areas the infiltrate was scarce and necrotic cells contained pale-stained myofibrillar material. Muscle obtained from control mice had a normal histological pattern with no evidence of myonecrosis or hemorrhage, and without an increase in the cellular infiltrate. Ultrastructurally, most inflammatory cells were macrophages or neutrophils. Some of these cells were performing phagocytosis of material within necrotic muscle cells (Fig. 3). Myofibrillar material, as well as mitochondria and vesicles, were located inside phagocytic vacuoles in macrophages and neutrophils.

DISCUSSION

Our results indicate that there is an abundant inflammatory infiltrate in skeletal muscle injected with *B. asper* venom. Inflammatory cells arrive at affected muscle several hr after myonecrosis develops. It has been described that *B. asper* venom induces drastic myonecrosis rapidly after envenomation (Gutiérrez *et al.*, 1980 a; Gutiérrez *et al.*, 1984). Inflammatory cells begin to increase in number in necrotic muscle six hr after injection, reaching their peak by 48 hr. As in other cases of inflammatory responses, polymorphnuclear leucocytes were more abundant in the early time intervals, whereas macrophages peaked at later time periods (Movat, 1979).

This pattern of inflammatory infiltrate composition is partially different from the ones observed in other examples of myonecrosis. For instance, in muscle damage induced by the local anesthetic bupivacaine and by plasmocid, the predominant inflammatory cells are macrophages (Nonaka *et al.*, 1983, 1984). In regard to snake venoms, an abundant phagocytic cell infiltrate after myonecrosis induced by the venoms of *Micrurus nigrocinctus* (Gutiérrez *et al.*, 1980 b) and *Oxyranus scutellatus* (Harris and Maltin, 1982), as well as by the toxins notexin and taipoxin (Pluskal *et al.*, 1978; Harris and Maltin, 1982) has been described. In the case of myonecrosis by notexin, Harris and Johnson (1978) observed that between 3 and 6 hr after injection, necrotic fibers had been invaded by polymorphonuclear leucocytes, although Pluskal *et al.* (1978) suggested the possibility that different cell types might migrate to necrotic tissue at different times. One common finding in all of these studies is that inflammatory infiltrate starts to accumulate several hr after the insult, despite the fact that myonecrosis is evident soon after injection of the myotoxic agents.

Some areas of necrotic muscle were almost devoid of inflammatory cells even one week after envenomation. In these areas, necrotic material had not been removed. Since *B. asper*

venom drastically affects the microvasculature, it may be that damage to blood vessels is partially responsible for this lack of infiltrate in some areas. In myonecrosis induced by toxins that affect muscle cells without inducing hemorrhage, necrotic tissue is cleared by phagocytosis in the first 2 to 3 days (Harris *et al.*, 1975; Harris and Maltin, 1982). Our observations may have implications on the issue of muscle regeneration, since clearing of necrotic tissue by phagocytosis is a requirement for regeneration to proceed.

Ishiura *et al.* (1984) have addressed the problem of myofibrillar degradation by muscle proteases in plasmocid-induced myonecrosis. They proposed a "two-step" mechanism in myofibrillar degradation. During the first 12 hr there is a limited hydrolysis of specific components, especially alpha-actinin, although the ultrastructural organization of myofilaments is severely affected. This early phase is responsible for only a minor myofibrillar degradation. After 12 hr, a macrophage-rich infiltrate accumulates in necrotic muscle, and lysosomal cathepsins derived from these inflammatory cells are responsible for a drastic degradation of myofibrillar proteins. Our results with *B. asper* venom agree with the "two-step" model, since electrophoretic studies showed that there was little, if any, myofibrillar degradation before 6 hr, although myofilaments were structurally affected at this period (Gutiérrez *et al.*, 1984). One the other hand, a drop in non-collagen proteins was observed by 48 hr, together with a decrease in the intensity of most myofibrillar proteins in electrophoresis. This protein degradation correlates with the presence of an abundant inflammatory infiltrate after 24 hr. Thus, the initial disorganization and clumping of myofilaments is not related to a generalized protein degradation, but probably to a limited alteration of structurally relevant components which regulate the assembly of myobrils. Therefore, their disturbance results in a loss of myofibrillar organization, without widespread protein hydrolysis. Later, the arrival of neutrophils and macrophages results in a more significant protein degradation.

On the basis of the abundant infiltrate observed, it is interesting to study the possibility that enzymes released from leucocytes or macrophages may cause further damage to muscle cells. It has been demonstrated that lysosomal enzymes of polymorphonuclear leucocytes, such as acid proteinases, collagenases and elastases, are responsible for tissue damage in inflammatory responses (Movat, 1979). However, in our case, there was not a decrease in CK contents of muscle after 6 hr. This implies that muscle necrosis was a rapid phenomenon, after which no further muscle cell death occurred. Thus, inflammatory cells played a role in degradation and clearing of necrotic cells, but not in the development of further muscle cell damage.

RESUMEN

Se estudió los cambios y la composición del infiltrado inflamatorio que se desarrolla en el músculo gastronemio de ratones a consecuencia de inoculaciones de veneno de serpiente "terciopelo" (*Bothrops asper*). El veneno produjo una severa y rápida mionecrosis, de acuerdo al análisis histológico y a la drástica disminución de los niveles musculares de la enzima creatina quinasa (CK). Se observó un escaso infiltrado inflamatorio a las 6 hr, pero hubo un aumento evidente a las 24, 48, y 72 hr. A las 6 y 24 hr el infiltrado presentó un predominio de leucocitos polimorfonucleares neutrófilos, en tanto que a las 48 y 72 hr se observó un aumento en el porcentaje de macrófagos. Histológicamente, las células inflamatorias se observaron en el interior de las fibras musculares necróticas, así como en el espacio intersticial; sin embargo, algunas áreas necróticas no contenían células inflamatorias. En un intento por correlacionar la presencia de células inflamatorias con la degradación de las proteínas miofibrilares, se observó muy poca degradación proteica a las 6 hr. Por otra parte, a las 48 hr se dio una disminución de las proteínas "no colágenas" del músculo, así como una disminución en algunos componentes miofibrilares, de acuerdo al análisis electroforético. Las enzimas proteolíticas presentes en células inflamatorias pueden jugar un papel importante en la degradación de las proteínas miofibrilares luego de mionecrosis inducida por el veneno de *B. asper*.

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