Muscle regeneration induced by snake venom. A histological and histochemical study

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Summary. This report describes the regeneration pattern of anterior tibial muscle of the rat after the inoculation of the snake venom of *Bothrops jararacussu*. The results show that this regeneration pattern is rather similar to the pattern described in other experimental models. Three days after the injection, three differentiated areas are established: a periferic one of surviving fibres, a second one called myogenic area, and the last one, more internal, made of necrotic fibres that are phagocited by macrophages. The surface of the surviving muscle fibres has myoblasts sticking to it and five days after, the myogenic area is occupied by many of them. Both the previous phagocytosis and the myoblasts came from the area of current uninjured fibres. After 30 and 60 days the regeneration is completed and there are only a few marks that show that the regeneration has taken place.

Key words: Skeletal muscle, Regeneration, Snake venom

Introduction

Many snake and scorpion venoms show significant myotoxic activity, which appears to affect the skeletal muscle fibre, causing lesions in the plasma membrane and subsequent increase in permeability to calcium and other ions, leading to the death of the cell (Gutierrez et al., 1984; 1986; Luque et al., 1987). In recent years, this miotoxic activity has been used to induce regeneration of the skeletal muscle (Klein-Ogus and Harris, 1983; Maltin et al., 1983; Queiroz et al., 1984).

This study deals with the regeneration pattern of the anterior tibial muscle of the rat following the intramuscular injection of venom from the snake *Bothrops*

jararacussu. The regeneration pattern observed is similar to that reported for the EDL muscle in the free graft described by Hansen-Smith and Carlson (1979).

Materials and methods

Male Wistar rats, weighing approximately 200 g, were anaesthetised with ether and injected directly in the centre of the anterior tibial muscle with 1 mg venom from *Bothrops jararacussu* (Sigma) dissolved in 0.05 ml physiological saline serum. Control rats were injected with the same quantity of physiological saline serum without venom. The rats were sacrificed at the following intervals after injection: 1, 2, 3, 4, 5, 7, 9, 15, 30 and 60 days.

The anterior tibial muscle was extracted from its anatomical compartment and samples were taken from the middle third of the muscle for experiment and control. Samples were rapidly frozen in isopenthane which had previously been cooled in liquid nitrogen. Serial sections of 8 μm thickness were obtained in a cryostat at -30° C.

Sections were stained with hematoxylin-eosin and modified Gomori trichrome. For the histochemical analysis, the following reactions were used: myofibrillar adenosine triphosphatase (ATPase) at pH 9.4, succinic dehydrogenase (SDH) and NADH-tetrazolium reductase. Alkaline phosphatase and acid phosphatase reactions were also used, together with the acridine orange fluorescent reaction for the identification of nucleic acids. Details of these techniques can be found in Dubowitz and Brooke (1973) and Sarnat (1983).

Results

Control muscles

Only two muscles, at 2 and 7 days, showed evidence of changes, including respectively the appearance of necrotic

fibres and one group of small regenerative fibres. These changes were insignificant, and may have been due to the mechanical trauma caused by the needle. No changes were found in the other control muscles; all fibres showing normal size and staining.

Experimental muscles

One day after the injection of the venom, two clearly different areas were evident: a large central region showing significant degeneration of muscle fibres, and a group of normal fibres which had survived the agression centred in peripheral areas of the muscle. Degenerated fibres were large and rounded, the sarcoplasm slightly stained by histological techniques. Histochemical activity rapidly diminished in the necrotic area and no reaction was observed for any of the histochemical techniques used. Muscle fibres in the surviving area, however, retained their histochemical activity and different fibre types could be distinguished.

After two days, phagocytosis of degenerated muscle cells was observed in a significant area of macrophages very close to the normal mucle fibres. This marked the beginning of cell mediated degeneration. Thus, three areas could be distinguished: 1. A peripheral zone composed of normal muscle fibres; 2. An intermediate area consisting of macrophages, identified by the acid phosphatase technique (Fig. 1); and 3. An inner area made up of numerous degenerating fibres which showed no evidence of phagocytosis.

Three days after the injection, a centripetal shift was observed in the macrophage zone advancing into the inner area of degenerating fibres. Three distinct areas could thus be identified (Fig. 2): 1. The peripheral zone made up of normal fibres, where an interesting phenomenon was observed: basophilic spindle cells were visible in the interstitial space and many of them were prominent on the surface of undamaged muscle fibres to which they adhered closely. These cells were assumed to be myoblasts and showed intense orange fluorescence in response to the acridine orange technique, which suggested a high RNA content (Fig. 3); 2. An underlying area containing a small number of macrophages and some spindle-cells like those observed in the previous area; and 3. The innermost area of the muscle showed substantial swelling, phagocyting the degenerated fibres. Considerable alkaline phosphatase activity among muscle fibres in the undamaged area closest to the second area was proof of the proliferation of capillaries (Fig. 4).

After five days, the intermediate area was occupied by a considerable proliferation of myoblasts, many of which had fused to form multinucleated syncitia. This intermediate area is described as myogenic, since regeneration was found to be taking place there.

Seven days after injection, the intermediate or myogenic area was found to contain myotubes stemming from the fusion of the myoblasts which had previously occupied the area. H-E analysis showed these to be basophilic (Fig. 5), and many of them had centrally positioned nuclei. Histochemical activity in these myotubes was

intense for the oxidative enzymes (SDH and NADH-tr), but only showed a moderate reaction to ATPase. They also showed alkaline and acid phosphatase activity in granular form. The acridine orange technique provoked intense orange fluorescence in these myotubes while central nuclei retained their yellow fluorescence.

After nine days, the myogenic area contained abundant regenerative fibres, which were very close to each other, while the connective tissue was distributed around them to form new fascicles. In the innermost area, there was only a small degree of phagocytosis of some fibres and regeneration had reached the area.

After 15 days, muscle fibres of a different size were observed in the regenerative areas, joined closely together and giving rise to splitting (Fig. 6). Fibres were no longer basophilic and colouring was normal. Nuclei were frequently found to be centralised. The histochemical techniques showed that fibre activity was normal. Split fibres were of the same histochemical type and different types of fibres had become apparent (Fig. 7).

30 and 60 days after injection, the experimental muscles, now fully regenerated, could only distinguished from control muscles by a series of changes (Fig. 8): 1. regenerated fibres were of normal size and staining, but retained noticeable basophilic stippling inside; 2. many mature regenerated fibres had centralised nuclei; 3. some regenerated fibres were found to have a clearly delimited empty space inside, corresponding to incomplete fusion during regeneration; 4. some atrophic fibres were visible, which may be non-innervated regenerated fibres. The histochemical pattern and the distribution of fibre type were fully restored and the two areas described for the anterior tibial muscle, one deep red the other shallow white, were easily distinguishable.

The sequence of changes observed in this study is shown schematically in Fig. 9.

Discussion

Bothrops jararacussu venom caused a rapid necrosis of muscle fibres, which was evident one day after injection, and in addition to acting directly on the muscle fibre, could also contribute to thrombosis and ischemia (Queiroz et al., 1984). To facilitate the study of muscle regeneration, the direct inoculation of venom was directed toward the central region of the anterior tibial muscle. Necrosis in the central region was massive, while a strip of peripheral fibres survived.

Necrosis was followed by regeneration of fibres over the following days, both processes starting in the vicinity of the undamaged muscle fibres. The alkaline phosphatase reaction showed numerous capillaries in this area, which was to be expected, since functioning blood vessels have to be present both for phagocytosis and successful regeneration (Carlson and Faulkner, 1983). Thus, the belt of macrophages swept the necrotic area shifting towards the centre of the anterior tibial muscle. The appearance of active macrophages both facilitates and stimulates regeneration (Aloisi, 1970).

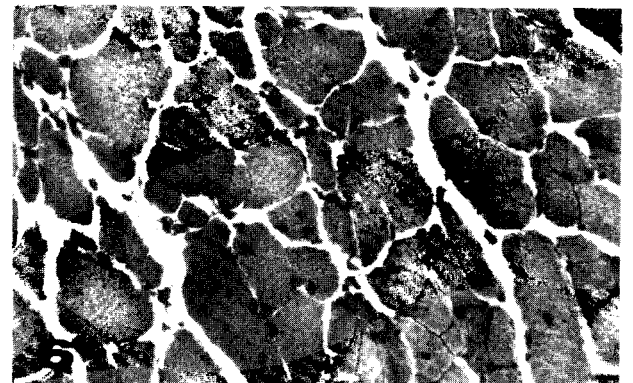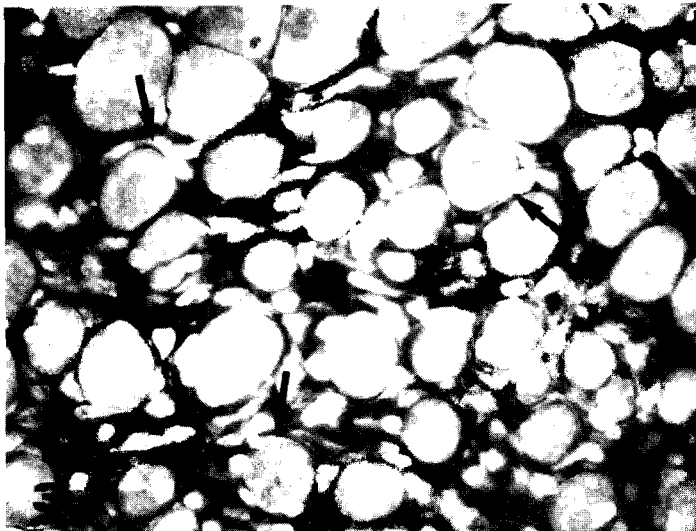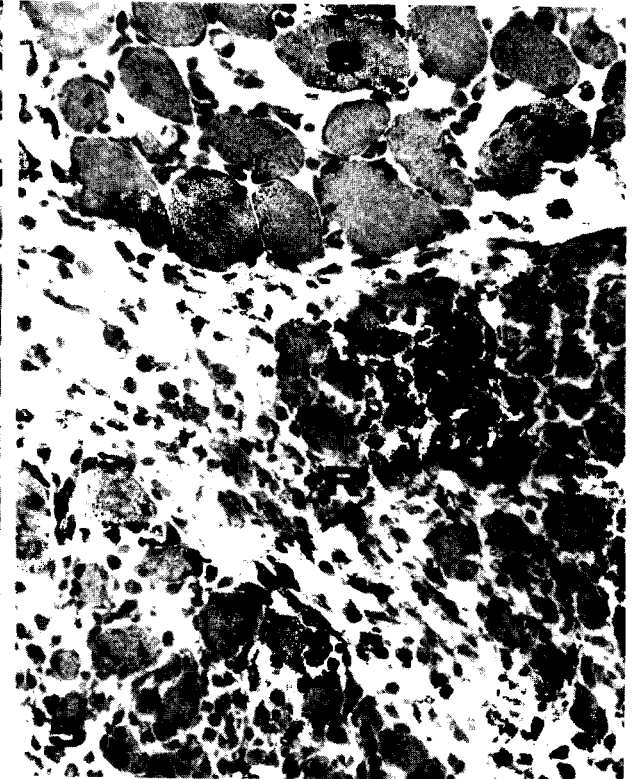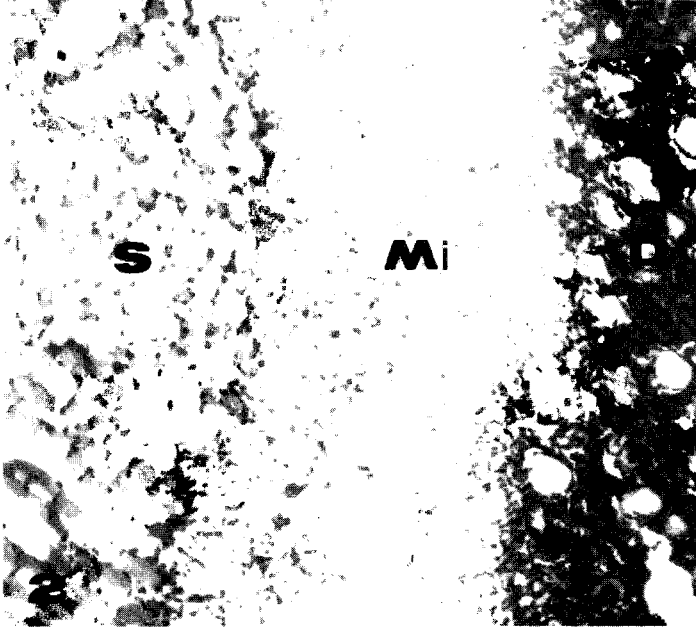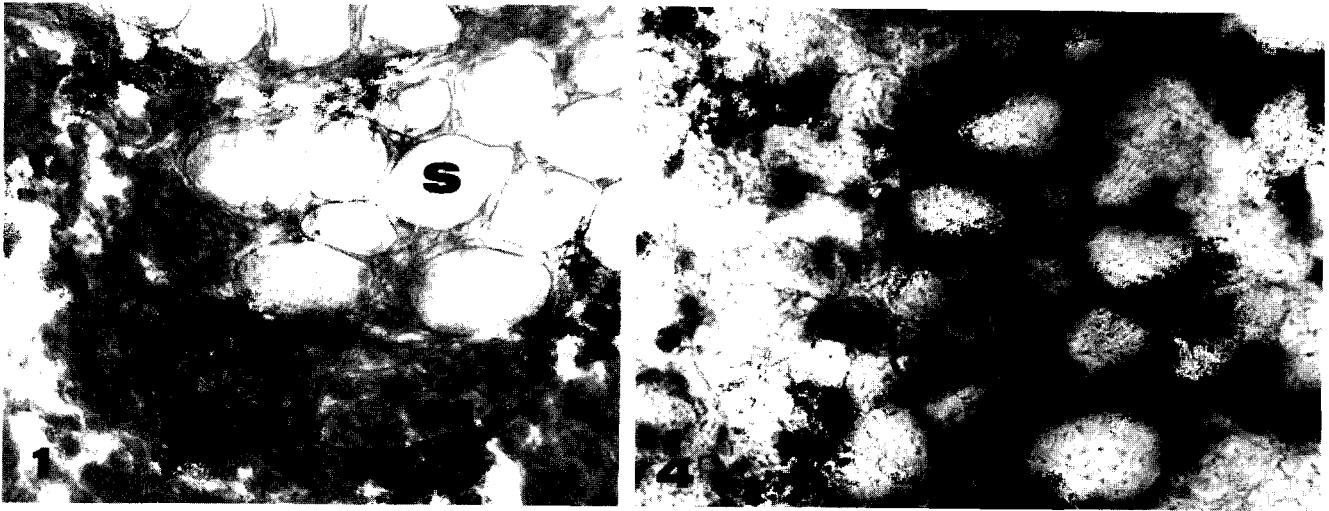Three distinct areas became evident when the

Fig. 1. Marked acid phosphatase activity in the region occupied by macrophages (M) which contrasts with the absence of activity in the fibres of undamaged area (S). × 250

Fig. 2. Section showing three different areas: an area of surviving muscle fibres (s), a myogenic area (Mi) and an area of degenerated fibres showing invasion of inflammatory cells (D). HE. × 100

Fig. 3. Assumed myoblasts with orange fluorescence in the interstitial

space. Some of these (arrows) adhere to the surface of normal muscle fibres. Acridine orange. × 250

Fig. 4. Note the considerable alkaline phosphatase activity on the edges of the surviving region. × 250

Fig. 5. Regenerative fibres occupying the myogenic area. Above these, some fibres from the undamaged muscle area are visible (S). HE. × 250

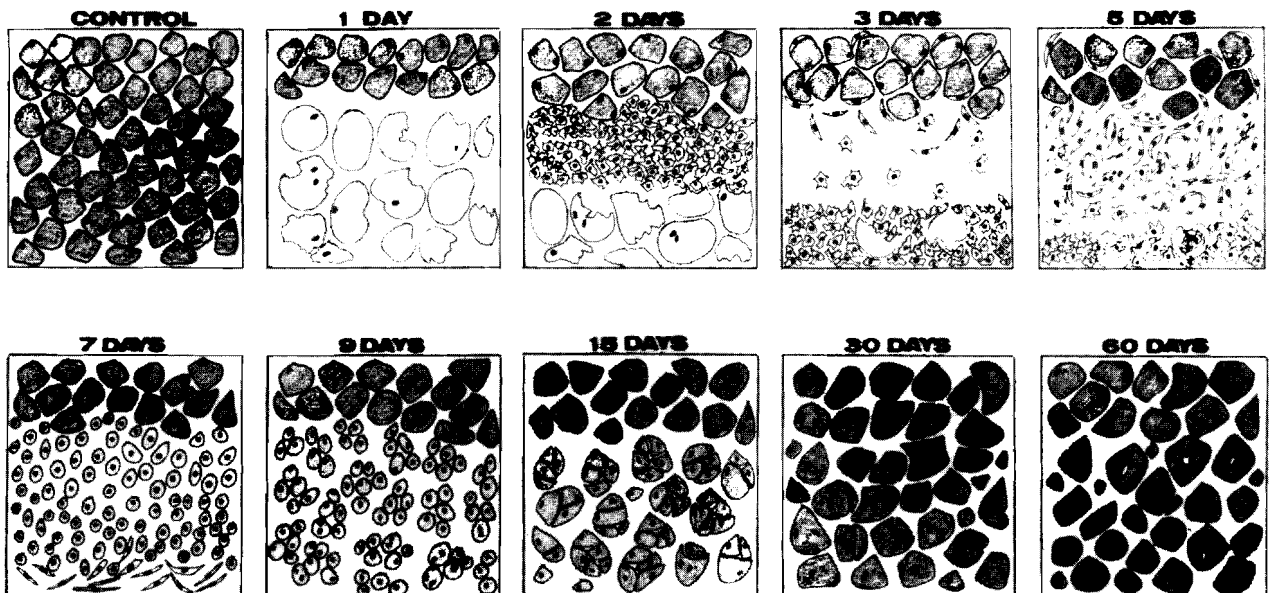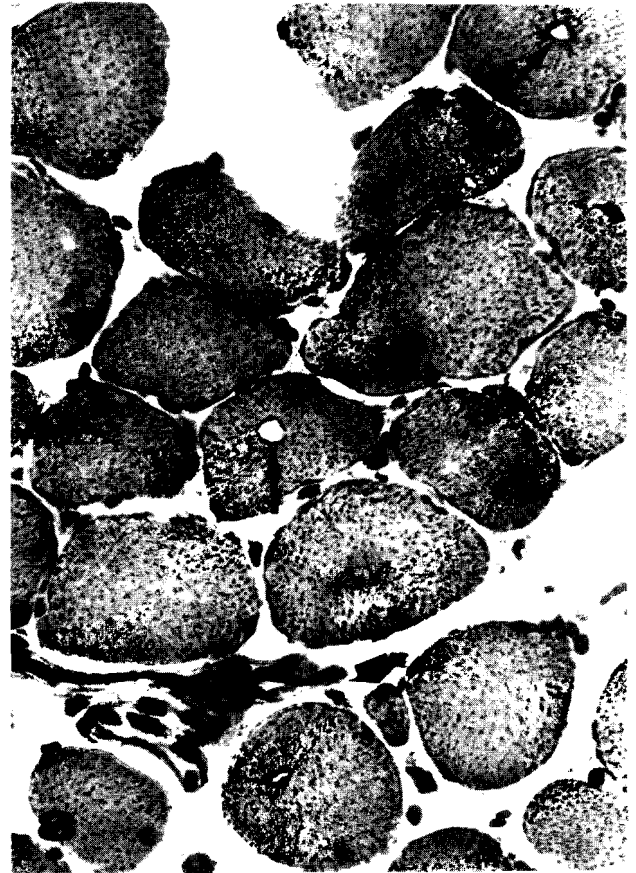**Fig. 6.** Section showing numerous split fibres. HE. × 250

Fig. 7. NADH-tr activity in surviving region (S) and regenerative area (R). Different fibre types are becoming apparent in the latter area. × 250

Fig. 8. Section of regenerated muscle, 60 days postinoculation. Regenerated fibres show a noticeable stippling in the sarcoplasm and

two fibres have empty spaces inside (thin arrows). An atrophic fibre is also visible in the interstitial space (thick arrow), together with another with a centralised nucleus. HE. × 400

Fig. 9. Schematic representation of the sequence of changes occurring during the regenerative process.

macrophage belt advanced into the area of necrotic remains. These three areas have also been observed in other experimental models used to study muscle regeneration, like local application of cold (Reznik and Engel, 1969), inoculation of mepivacain (Basson and Carlson, 1980), muscle transplant (Hansen-Smith and Carlson, 1979; Gulati, 1985) and in the regeneration of human muscles suffering infarcts (Engel, 1979).

The most interesting aspect of this study was probably the appearance in the area of undamaged fibres of numerous spindle-cells; they were often found adhering to the surface of muscle fibres. Their characteristic sarcolemic location, adjacent to fibre surface suggests that these may be activated satellite cells (Mastaglia and Kakulas, 1970). Many spindle cells similar to these were found in the interstitial space of the intact region and were assumed to be myoblasts. This assumption would need to be confirmed by a subsequent electron microscopy study. The formation of myoblasts in the area of surviving fibres has been reported by Hansen-Smith and Carlson (1979) three days after the free graft of the EDL muscle.

On the fifth day after inoculation of venom, evidence of a marked proliferation of myoblasts in the myogenic area suggests that they have passed into that area from the intact region. If satellite cells in the centre of the muscle do not survive to iniciate the process of regeneration, then the formation of new fibres might originate in the area of normal fibres whence waves of myoblast leave to initiate the formation of new fibres. This theory is supported by the results of studies carried out by Klein-Ogus and Harris (1983) who report that satellite cells in non-necrotic fibres react to lesion produced elsewhere in the muscle. This migratory capacity thus allows them to be recruited in other parts of the muscle. Some authors have reported that satellite cells are found to be activated under electron microscopy and in some cases appear to move beyond the original fibre to the extrafibrillar space (Maltin et al., 1983).

The sequence of changes occurring later in the myogenic area is similar to the process reported in embryonic myogenesis (Allam, 1979; Carlson and Faulkner, 1983) giving rise to multinucleate tubular structures described as myotubes. This sequence of changes is the same as that described in other studies of regeneration in mammals. Most regenerative fibres appear after 7 and 9 days, giving rise to the formation of mature muscle fibres after 30 or 60 days. The histological and histochemical characteristics of these regenerative fibres concur with those reported by other authors. The numerous split fibres visible at 15 days postinoculation appear to be a result of the fusion of various myotubes to form a single muscle fibre. This would appear to be the final destiny of the split fibres, since fewer such fibres were observed at 30 days and none at all at 60 days. Split fibres may thus be considered as one stage in the whole process of muscle fibre regeneration, and have been studied in depth by Schmalbruch (1979). Completely regenerated muscles can be distinguished from control muscle by the presence of mature fibres with centralised nuclei, which is evidence that regeneration has taken place (Carlson and Rainin,

1985). Although the muscles in which regeneration was complete showed other changes, such as atrophic fibres and intrasarcoplasmic spaces where fusion was incomplete, these were considered to be side-effects of regeneration.

According to the regeneration pattern described in this study, regeneration originates in the undamaged area, possibly because all the satellite cells in the degenerated area have died, although this has not been confirmed by electron microscopy. Queiroz et al. (1984) report that the fibrosis and deterioration of regeneration after inoculation with *Bothrops jararacussu* venom is due to the destruction of satellite cells by the venom and/or ischemia. Damage to microcirculation in the necrotic area gives rise to the centripetal regeneration pattern due to the growth of the intact microcirculation from the undamaged area. This pattern evidently requires the reestablishment of circulation in the damaged muscle in order for regeneration to advance.

In short, this study shows that the pattern of regeneration of the anterior tibial muscle following inoculation with *Bothrops jararacussu* venom is centripetal, and is very similar to the pattern found in other studies of experimental regeneration. Our observations indicate considerable myogenic potential in the area occupied by undamaged fibres, and suggests that satellite cells from intact fibres may be the source of myoblasts which give rise to the regeneration of damaged areas of the skeletal muscle. This possibility has also been suggested by other authors (Klein-Ogus and Harris, 1983; Maltin et al., 1983). Recently it has been reported that ischemic central areas in the freely grafted muscle are regenerated by precursor cells located outside of the ischemic zone (Phillips et al., 1987).

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