Int. J. Exp. Path. (1991), 72, 211–226 ADONIS 095996739100020Q

Degenerative and regenerative changes in murine skeletal muscle after injection of venom from the snake *Bothrops asper*: a histochemical and immunocytochemical study

Vilma Arce*, Fernando Brenes† and José María Gutiérrez‡
**Centro de Investigación en Biología Celular y Molecular, Universidad de Costa Rica; †Laboratorio de Patología Experimental, Hospital México, CCSS; ‡Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica*

Received for publication 25 May 1990
Accepted for publication 21 November 1990

Summary. The degenerative and regenerative changes in murine skeletal muscle after injection of *Bothrops asper* venom were studied by histological, lectin histochemical and immunocytochemical techniques. According to our observations, the process was divided into four main stages:

(a) During the first 3 days prominent degenerative events took place in skeletal muscle fibres, capillaries, arteries, veins and intramuscular nerves. An inflammatory infiltrate was abundant after the first day and removal of necrotic material was well advanced by the third day.
(b) Muscle regeneration was evident by the fourth day. From 4 to 6 days there were two populations of regenerating muscle fibres, one of apparently normal fibres located in areas where capillary vessels were abundant, and another population of groups of regenerative fibres showing signs of degeneration. This second type of fibre was predominant in areas where the number of capillaries was greatly reduced.
(c) One and 2 weeks after envenomation areas of small regenerative fibres of normal morphology and areas of degenerating regenerative fibres were observed. The latter were abundant in regions of dense fibrotic tissue and scarce capillaries.
(d) Finally, at 4 and 8 weeks after envenomation there were both areas of fibrosis and areas where regenerating muscle fibres predominated. However, the diameter of these fibres was abnormally small, an indication that they may have been atrophic fibres.

It is suggested that muscle regeneration is partially impaired after myonecrosis induced by *Bothrops asper* venom, probably due to the damage induced by this venom on muscle microvasculature and nerves.

Keywords: muscle regeneration, *Bothrops asper* venom, immunocytochemistry, lectins, myonecrosis

Correspondence: Dra. Vilma Arce, Centro de Investigación en Biología Celular y Molecular, Universidad de Costa Rica, San José, Costa Rica.

Skeletal muscle regeneration has been described, both clinically and experimentally, after muscle fibre damage induced by a variety of injuries (Allbrook 1981; Plaghki 1985). In some cases, for example after myonecrosis induced by local anaesthetics (Nonaka *et al.* 1983) and by some snake venom myotoxins (Harris & Maltin 1982; Gutiérrez *et al.* 1984b, 1989; Homsi-Brandeburgo *et al.* 1988), regeneration proceeds to completion. In these conditions there is no damage to muscle-associated vasculature or to peripheral nerves. Thus, these two basic requirements for regeneration (Allbrook 1981; Plaghki 1985) are not affected.

In the case of crotaline snake venoms which induce prominent local and systemic alterations, there is damage not only to muscle fibres, but also to vasculature (Tu 1977; Ohsaka 1979). In these circumstances, muscle regeneration is only partial, as has been described in the case of myonecrosis induced after inoculations of venom of the snake *Bothrops asper* (Gutiérrez *et al.* 1984b; 1986). This species is responsible for the large majority of bites in Central America (Bolaños 1982), and these envenomations are followed by a conspicuous muscle tissue loss at the region of venom injection, due to a deficient regenerative process. On the basis of the relevance of this pathological phenomenon and the possibility of using it as a model to study impaired muscle regeneration, a histological, histochemical and immunocytochemical study was performed in order to gain a better understanding of the degenerative and regenerative changes in murine skeletal muscle following injection with this venom.

Materials and methods

Snake venom

Crude *Bothrops asper* venom was taken from a pool obtained from adult specimens collected in the Atlantic region of Costa Rica. Once obtained, venoms were lyophilized and stored at −30°C.

Animals

Groups of Webster mice (18–20 g) were injected intramuscularly in the right gastrocnemius muscle with 50 μg of crude venom (dissolved in 100 μl of phosphate-buffered saline solution, pH 7.2). Mice were maintained on standard laboratory diet and water *ad libitum*.

Tissue preparation

Mice were sacrificed by cervical dislocation and muscle tissue samples from injected gastrocnemius were collected at several time intervals: 1, 2, 3, 4, 5 and 6 days, 1, 2, 4 and 8 weeks. Groups of three mice were used for each time interval. Uninjected gastrocnemius muscle from one of the animals was collected and used as control for each time interval. In addition, four mice were injected as described with 100 μl of saline solution, and samples were obtained 24 h after injection.

One half of the muscle was fixed in BG-$HgCl_2$, a solution containing 6% $HgCl_2$, 1% sodium acetate and 0.1% EM grade glutaraldehyde (Merck, FR Germany) (Schulte & Spicer 1983a, b). The other half was fixed in modified Zamboni fixative (2% paraformaldehyde in 0.1 M Sorensen phosphate buffer containing 15 ml saturated picric acid solution, pH 7.4) (Pearse 1980). Tissues were fixed for 6 h at room temperature, dehydrated in alcohols, embedded in paraffin and serial sections were cut at 5 μm. Mercury was removed from the sections with Lugol's solution prior to all staining procedures.

Histology

For the morphological analysis of tissue sections, a modified Masson trichrome stain was used (Arce-Arenales 1986).

Measurement of muscle fibre diameters

The diameters of a total of 200 fibres were measured in each experimental group and

the mean and s.d. were obtained. A regenerating fibre was defined as a muscle fibre with centrally-located nuclei and with cytoplasmic basophilia. This basophilia was more evident in regenerating fibres from samples obtained during the first 2 weeks after envenomation. The significance of the differences between the means of groups was determined using a *t*-test, comparing the calculated value to the Z table, because of the sample size.

Histochemical staining procedures

Methyl green–pyronin. Sections were washed 10 times with distilled and deionized water and were subsequently incubated with methyl green–pyronin solution (Sigma Chemical Company, St Louis, MO, USA) during 7.5 min at 22°C. Afterwards, these sections were washed with 10 rinses of distilled and deionized water, air dried, cleared and mounted. This method stains DNA green-blue and RNA red (Stevens & Bancroft 1982).

Lectins. The lectins used in this study along with their reported carbohydrate binding specificities are listed in Table 1. Tissue sections fixed in BG-$HgCl_2$ were used for lectin histochemistry. Biotinylated lectins from wheat germ (WGA), concanavalin A (Con A) and *Ulex europaeus* (UEA_1) were purchased from Sigma Chemical Company. Briefly, the sections were incubated with a 10-μg/ml solution of lectin in 0.05 M Tris buffer, pH 7.2 containing 0.1 mM $MnCl_2$, $MgCl_2$, $CaCl_2$ for1 h at room temperature, and rinsed in TBS (0.05 M Tris buffered saline, pH 7.2). Subsequently, tissue sections were incubated with streptavidin–horseradish peroxidase conjugate (Sigma) at a concentration of 5 μg/ml for 1 h at room temperature, rinsed in TBS, and developed with diaminobenzidine–H_2O_2 (Sigma) substrate solution, and counterstained with Carazzi's haematoxylin (Stevens 1982).

Controls for lectin staining included incubation of sections with a 10-μg/ml lectin solution containing 0.1 M of the following monosaccharides (Sigma), *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, or *N*-acetylneuraminic acid type VIII for WGA staining and 1-*O*-methyl-α-D-glucopyranoside, or

Table 1. Lectins used in this study

Lectin (common name) abbreviation	Carbohydrate specificity	References
Triticum vulgaris (wheat germ) WGA	D-GlcNAc> Neu5Ac> D-GalNAc	Burger and Goldberg (1967) Allen *et al.* (1973) Bhavanandan and Katlic (1979) Peters *et al.* (1979) Monsigny *et al.* (1980)
Canavalia ensiformis (jackbean) Con A	α-D-MM> α-D-MG>	Goldstein *et al.* (1965)
Ulex europaeus (gorse seed) UEA_1	α-L-Fuc	Allen *et al.* (1977) Goldstein and Hayes (1978) Pereira *et al.* (1978)

Abbreviations: GlcNAc, *N*-acetylglucosamine; Neu5Ac, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; α-D-MM, α-methyl-D-mannoside; α-D-MG, 1-*O*-methyl-α-D-glucopyranoside; α-L-Fuc, α-L-fucose.

methyl-α-D-mannopyranoside for Con A, and α-L-fucose for UEA_1. Sialic acid was cleaved enzymatically by incubating sections for 18 h at 37°C in a 0.2 U/ml solution of *Vibrio cholerae* sialidase (Calbiochem, La Jolla, CA, USA) in 0.1 M acetate buffer, pH 5.5 containing 1 mg/ml $CaCl_2$. Some sections were digested with α-amylase for 1 h at 22°C prior to staining with Con A or PAS (Cook 1982) at a concentration of 1 mg/ml in 0.02 M sodium potassium phosphate buffer, pH 6.0 in order to demonstrate the presence of glycogen (Cook 1982).

Antibodies. Tissue sections fixed in Zamboni were used for immunocytochemical studies. All dilutions for the sera used were made in 0.05 M Tris buffered saline, pH 7.6. Briefly, sections were digested enzymatically with 0.01% trypsin in 0.1% $CaCl_2$, pH 7.6 for 10 min, and then incubated with 5% normal serum of the species from which the secondary antibody was raised to block non-specific tissue staining. Subsequently, they were incubated with several primary antisera; anti-S-100 1:300, anti-desmin 1:80 (both rabbit immunoglobulins from Dakopatts, Denmark) and anti-Factor VIII, chicken immunoglobulins 1:200 (Immunosystem AB, Sweden). Anti-S-100 was used because it has been shown to stain nerve bundles in human tissue, thus providing a way to study the integrity of nerve bundles after envenomation (Barwick 1990a). Anti-desmin stains Z line interstices of skeletal muscle (Barwick 1990b), and staining is lost after myotoxin-induced muscle necrosis (Gutiérrez *et al.* 1990). Finally, anti-Factor VIII was used since it stains endothelial cells in human tissue (Hoyer *et al.* 1973). Biotinylated goat anti-rabbit, 1:500 (Sigma) and biotinylated goat anti-chicken, 1:500 (Janssen Life Sciences Products, Belgium) were used for 1 h. Finally, sections were incubated for 1 h with streptavidin–peroxidase, developed and counterstained as described previously. The control procedure for immunocytochemical staining included omission of the primary antibody and replacement with rabbit normal serum. This control procedure resulted in loss of specific staining.

Quantitation of hydroxyproline content of envenomated muscle

Changes in collagen were followed by quantitating muscle hydroxyproline contents (López de León & Rojkind 1985). Eight groups of four mice each (18–20 g bodyweight) were injected intramuscularly in the right gastrocnemius muscle with 50 μg of *Bothrops asper* venom (dissolved in 100 μl of phosphate-buffered saline solution, pH 7.2). At the time intervals of 2, 3, 4 and 5 days, 1, 2, 3, 4 and 8 weeks, groups of four or five mice were sacrificed by cervical dislocation and both gastrocnemius muscles from each animal were excised, weighed and hydrolysed by incubation with 6 M HCl at 110°C for 18 h. Dried samples were then resuspended in 0.5 ml of 2 M HCl, neutralized to pH 5–6 with NaOH, and the volume adjusted to 2.0 ml with distilled water. The hydroxyproline content of the samples was determined according to Prockop and Udenfriend (1960). The amount of hydroxyproline per gram of tissue was calculated and the results were expressed as a percentage, taking as 100% the hydroxyproline content of the non-injected gastrocnemius muscle.

Results

Lectin histochemistry and immunocytochemistry of control gastrocnemius muscle

Results obtained with WGA staining and control procedures are summarized in Table 2. No staining differences of histological sites from controls obtained at several time intervals after venom injection were observed. Positive staining with this lectin was totally abolished, after digestion with sialidase in intracytoplasmatic granules of muscle fibres, macrophages and polymorphonuclear leucocytes and remained in endomysium, nerve bundles, fibroblasts and endothelial cells.

Table 2. Comparative staining of several histological sites of gastrocnemius muscle with WGA prior and after several control procedures

Histological site and cell type	Histochemical staining method				
		WGA after or with			
	WGA	Sialidase	GluNAc	Neu5Ac	GalNAc
Myofibres					
intracytoplasmatic granules	4	0	0	4	4
endomysium	4	4	0	0	4
Nerve bundles					
axon	3	3	0	3	3
neurilemma	3	3	0	3	3
perineurium	3	3	0	3	3
Endothelial cells	4	4	0	0	4
Macrophages	4	0	0	4	4
Polymorphonuclear leucocytes	4	0	0	4	4
Fibroblasts	4	4	0	4	4

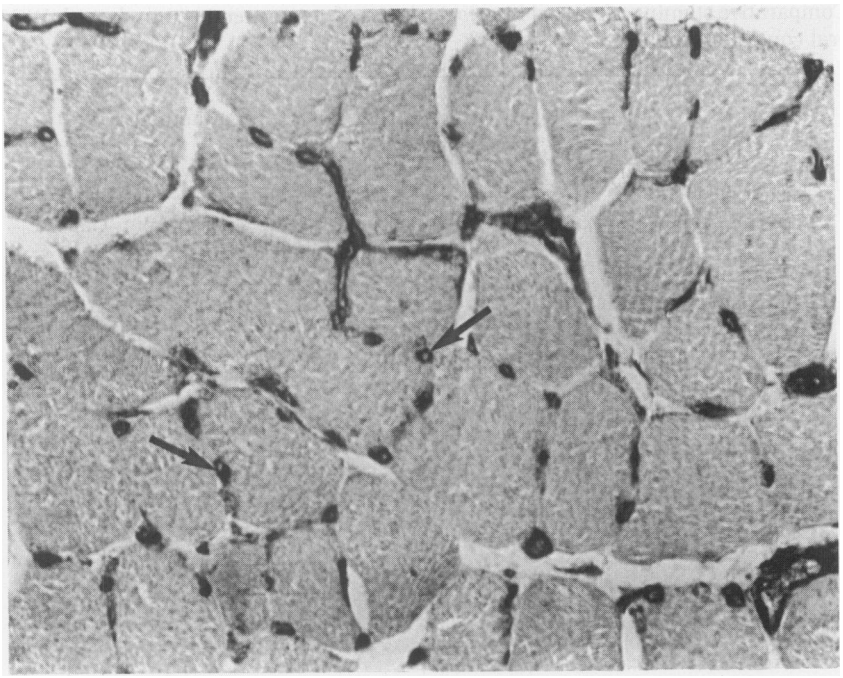
WGA, wheat germ agglutinin; Neu5Ac, *N*-acetylneuraminic acid; GluNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

Numbers indicate staining intensity on a subjectively estimated scale from 0, unreactive, to 4, most reactive.

Incubation of this lectin with *N*-acetylglucosamine abolished staining in all sites, while incubation with *N*-acetylneuraminic acid abolished positive staining in endomysium and endothelial cells. Staining was not abolished in any of the sites studied with *N*-acetylgalactosamine. Con A staining had a uniform general distribution in the muscle fibre cytoplasm, which was abolished after incubation with 1-*O*-methyl-α-D-glucopyranoside, but was unchanged after incubation with methyl-α-D-mannopyranoside. This staining was also abolished after enzymatic digestion with α-amylase. Endothelial cells of capillaries and larger blood vessels were stained with WGA, both before and after sialidase digestion (Fig. 1), whereas they remained unstained with UEA_1. The antibody against factor VIII-related antigen stained larger vessels but failed to stain capillaries.

Histological and histochemical changes after venom injection

Mice injected with saline solution showed the same histological and histochemical staining pattern as that observed in gastrocnemius muscle from control, uninjected mice. Intramuscular injection of *Bothrops asper* venom induced a complex series of acute degenerative alterations in muscle fibres, as well as in intramuscular nerves and blood vessels. Then, after the fourth day, degeneration was followed by a regenerative process. Major pathologic alterations taking place after envenomation were divided into the following stages: (a) acute degenerative changes (1–3 days after envenomation); (b) early regenerative changes (4–6 days); (c) further muscular and vascular regeneration and fibrosis (1–2 weeks); and (d) final stages in muscle fibre atrophy (4–8 weeks).



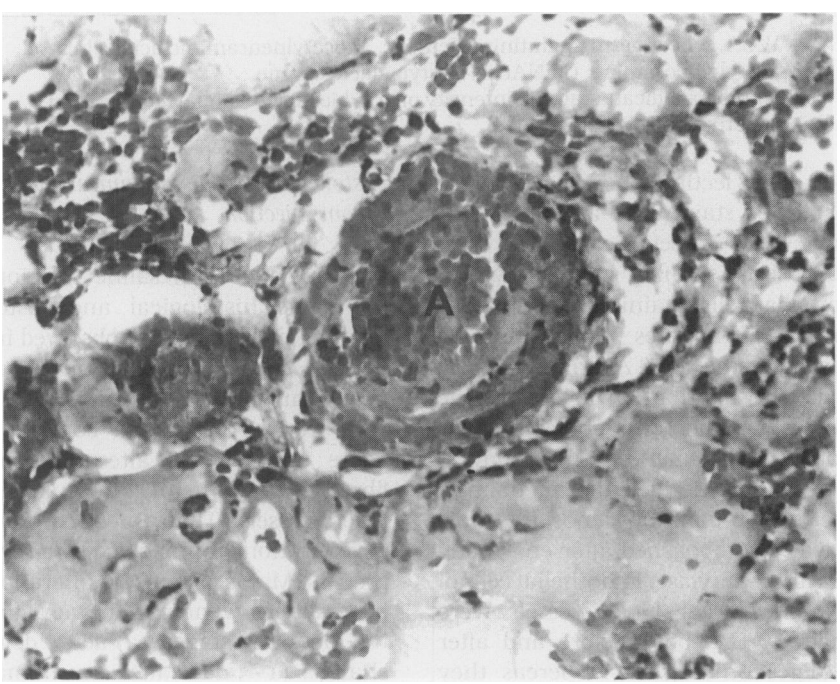


Fig. 1. Positive staining of endothelial cells (arrows) in a control, uninjected gastrocnemius muscle. WGA × 1600.

Fig. 2. Thrombosis of an artery (A) 1 day after venom injection. There is myonecrosis and haemorrhage as well as inflammatory infiltrate. Trichrome × 1600.

Acute degenerative changes (1–3 days after envenomation)

Widespread necrosis was observed in muscle fibres with progressive loss of sarcoplasm leaving in some cases an empty cellular space delimited by the basal lamina. These fibres showed greatly reduced to total loss of PAS, and anti-desmin staining. Vascular damage was observed with WGA and modified Masson trichrome stain (Fig. 2). This was characterized by oedema, thrombosis, vascular wall and endothelial cell disruption, as well as haemorrhage. Staining of the endothelium of capillaries, venules, arterioles, arteries and veins, and tunica adventitia of the last three blood vessels was lost progressively with ensuing necrosis until no staining was observed in advanced stages of necrosis (Table 3). This was accompanied by extensive inflammatory infiltrate, particularly composed of polymorphonuclear leucocytes. Nerve bundle damage observed with anti-S-100 staining showed axon loss, clumping of

Table 3. WGA staining of blood vessels in normal, regenerating and necrotic muscle

Histological site and cell type	Areas		
	Normal[a]	Necrotic[b]	Regenerating
Small arteries			
endothelial cells	4	0–4[c]	4[e]
elastica interna	0	0	0
tunica media	0	0	0
tunica adventitia	1	0–1[c]	1
Arterioles			
endothelial cells	4	0–2[c]	3[e]
elastica interna	0	0	0
tunica media	0	0	0
tunica adventitia	3	0–2[b]	3
Veins			
endothelial cells	4	0–3[c]	3[e]
tunica medica	0	0	0
tunica adventitia	2	0–3[c]	0
Venules			
endothelial cells	4	0–3[c]	3
Capillaries			
endothelial cells	4	0–2[c,d]	4[f]

Numbers indicate staining intensity on a subjectively estimated scale from 0, unreactive, to 4, most reactive.

[a] 'Normal' refers to areas of muscle tissue where there was no damage to muscle fibres, vessels and nerves; 'necrotic' refers to areas with necrotic muscle fibres 24 and 48 h after envenomation; 'regenerating' refers to areas with regenerating muscle fibres 4 days and 1 week after venom injection.

[b] No staining was observed in any histological site in advanced stages of necrosis.

[c] Staining continuity of morphological structure was lost.

[d] Greatly reduced number of capillaries in necrotic area.

[e] Increased number of endothelial cells and thickened wall.

[f] Increased number of capillaries.

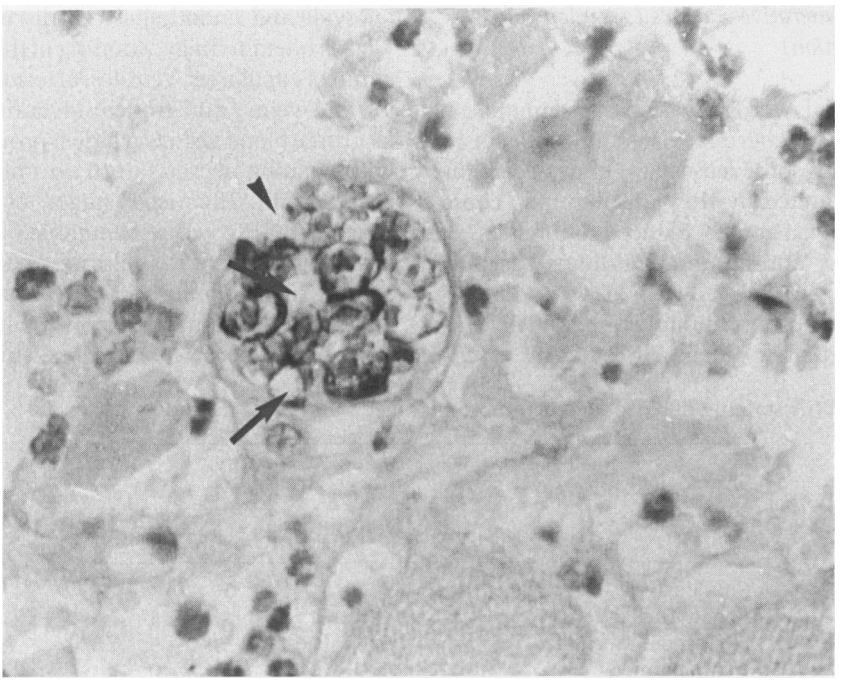


Fig. 3. Nerve bundle damage 1 day after venom injection: loss of axons (arrows) and loss of perineurium integrity (arrowhead). Anti-S-100 × 3000.

neurilemma and loss of perineurium integrity, seen as loss of staining continuity (Fig. 3). There was almost complete loss of nervous tissue in the area studied 2 days after venom inoculation, leaving S-100 stained fragments without any histological definition. Mononuclear inflammatory cells were observed within nerve fibres. The aponeurosis sheath was preserved but showed oedema and inflammatory cell infiltration.

Early regenerative changes (4–6 days after envenomation)

From 4 to 6 days, regenerative muscle fibres were observed. These were characterized by their small size and their central nuclei. Two populations of regenerating muscle fibres were recognized. One consisted of healthy fibres having preserved sarcoplasm, and small, intensely stained nuclei with apparently intact membrane. These fibres stained strongly with PAS and Con A. This staining was lost after digestion with α-amylase, indicating the presence of glycogen. Desmin stained these fibres in the Z line interstices of myofibrils. Capillaries having a normal morphological appearance were found surrounding these fibres, and there were relatively few adjacent macrophages (Fig. 4). Small arteries, arterioles, and veins found in this area showed an increased number of endothelial cells and a thicker wall, and presented a staining pattern with WGA similar to those found in the control area (Table 3).

The other population of regenerating fibres showed signs of degeneration including faintly stained large nuclei with loss of nuclear membrane and, in some cases, nucleoli. These areas were characterized by a diminished number of capillaries and a relatively large number of macrophages were observed surrounding the fibres (Fig. 5). These fibres showed very weak glycogen staining. Desmin staining was not altogether

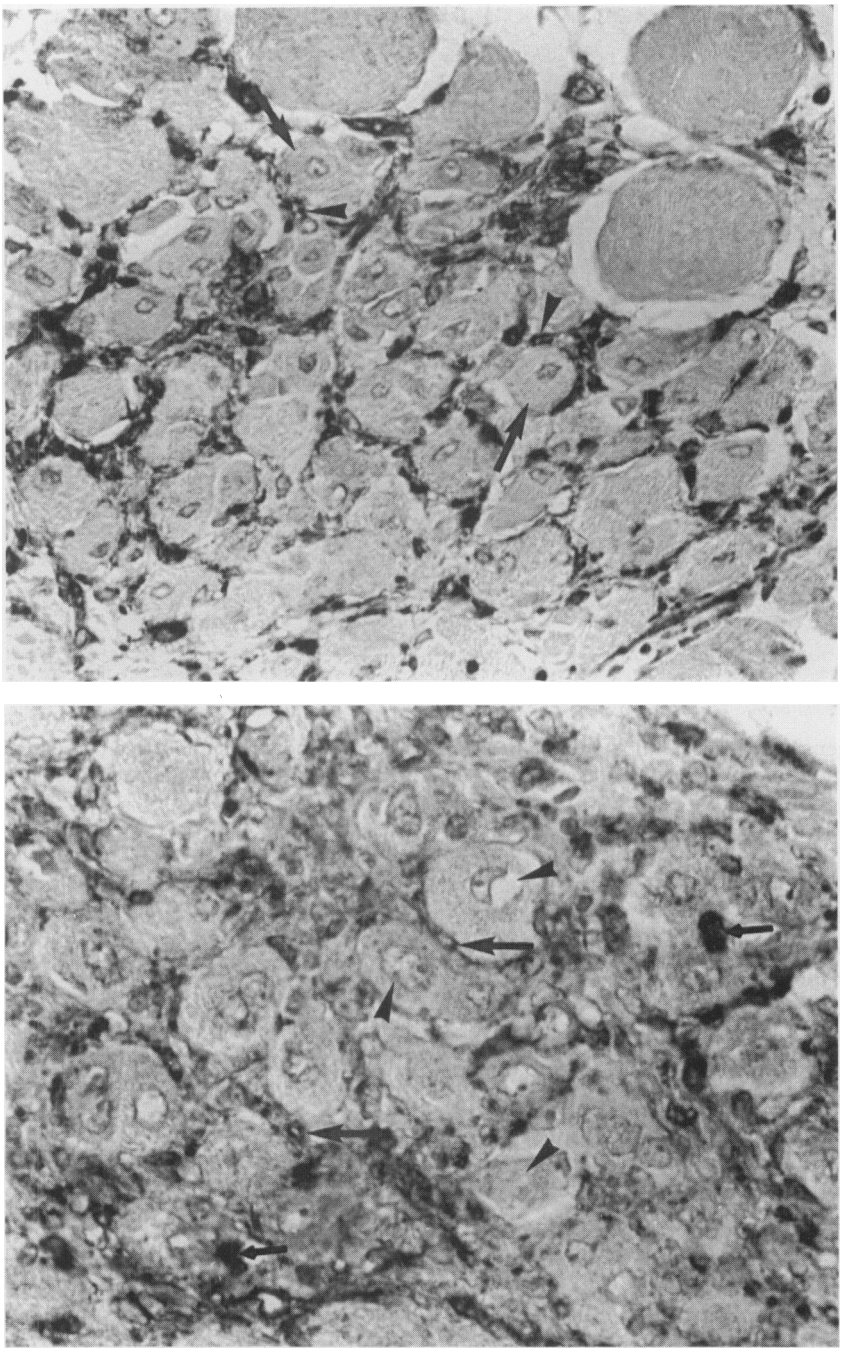


Fig. 4. Area of adequate muscle regeneration 6 days after venom injection. Regenerating muscle fibres show normal morphology (arrows) and are surrounded by capillaries (arrowheads). There are relatively few inflammatory cells. WGA × 1600.

Fig. 5. Area of impaired muscle regeneration 6 days after venom injection. Notice the sparcity of capillary vessels (arrows) around regenerating muscle fibres and the abundance of macrophages (small arrows). Regenerating muscle fibres show signs of necrosis (arrowheads), including large nuclei, or total loss of nuclei, and cytoplasmic vacuolization. WGA × 2000.

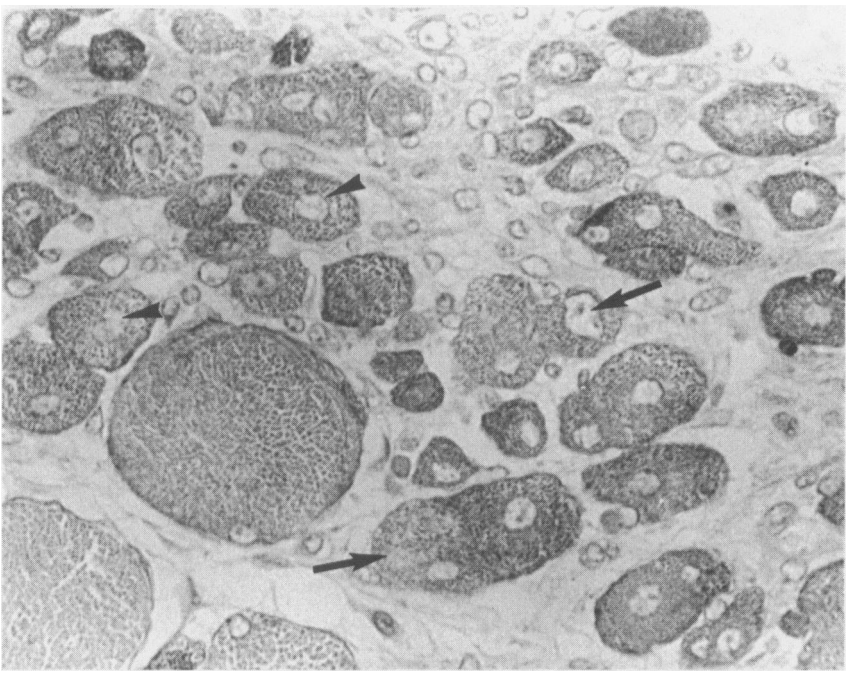


Fig. 6. Regenerating muscle fibres 4 days after venom injection showing signs of necrosis with focal loss of cytoplasmic staining (arrows) and signs of degenerating nuclei (arrowheads). Anti-desmin × 1600.

lost, but disclosed focal loss of cytoplasmic staining (Fig. 6). Desmin positive staining bore little correlation with the preservation of normal morphology, that is, myofibres with the morphological characteristics of degeneration described above still showed focal positive staining of the myofibrils. Total loss of desmin staining was rarely observed, and was found in fibres in a very late stage of necrosis, having only remnants of nuclear material and hyalinization of the cytoplasm. Vasculature in this area showed the same damage and histochemical staining pattern as described above for the acute degenerative changes.

Methyl green–pyronin staining was observed in healthy regenerating fibres but was absent in adult, normal myofibres. Methyl green stained nuclei, while cytoplasm and nucleoli stained with pyronin. Reduced staining intensity was observed in regenerating fibres showing signs of degeneration. Total absence of positive staining was observed only in very late stages of necrosis showing the same morphological characteristics described above.

Further muscular and vascular regeneration and deposition of collagen (1–2 weeks after envenomation)

There was a conspicuous deposition of collagen 1 and 2 weeks after venom injection, in agreement with quantitation of hydroxyproline which peaked at these two time intervals (Fig. 7). The process of necrosis of regenerating fibres in some areas of the muscle continued its course during this stage. Two zones of granulation tissue could be distinguished. At the periphery, regenerating fibres embedded in a loose collagenous matrix had the appearance of healthy looking ones with presence of proliferating capillaries and larger vessels with irregular walls, while regenerating fibres embedded in a dense collagenous matrix in a central zone

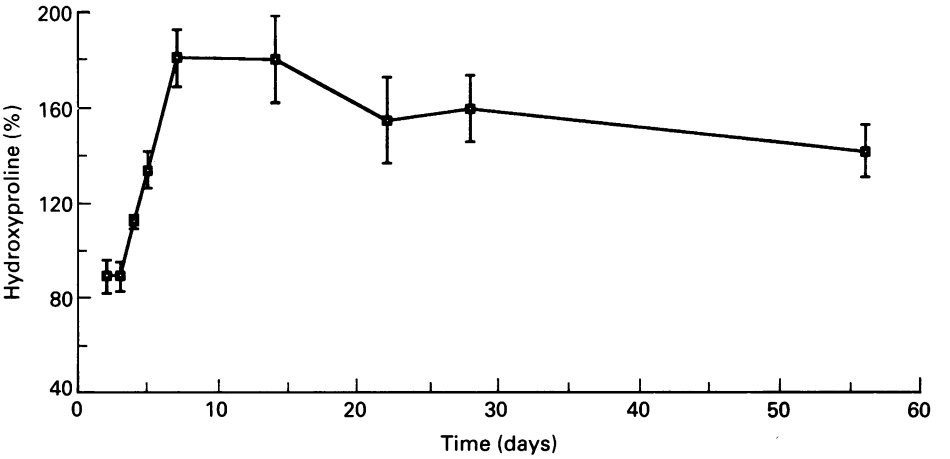


Fig. 7. Changes in hydroxyproline content of mouse gastrocnemius muscle at various time intervals after injection of *Bothrops asper* venom. Results are presented as a percentage taking as 100% the hydroxyproline content of non-injected contralateral gastrocnemius muscle.

showed signs of necrosis (Fig. 8), without large vessels and capillaries surrounding them. At 1 week, the large majority of regenerating muscle fibres had a diameter between 10 and 19 μm and by 2 weeks the diameter of regenerating muscle fibres had slightly increased (Fig. 9, Table 4).

Nerve bundle damage was still observed 1

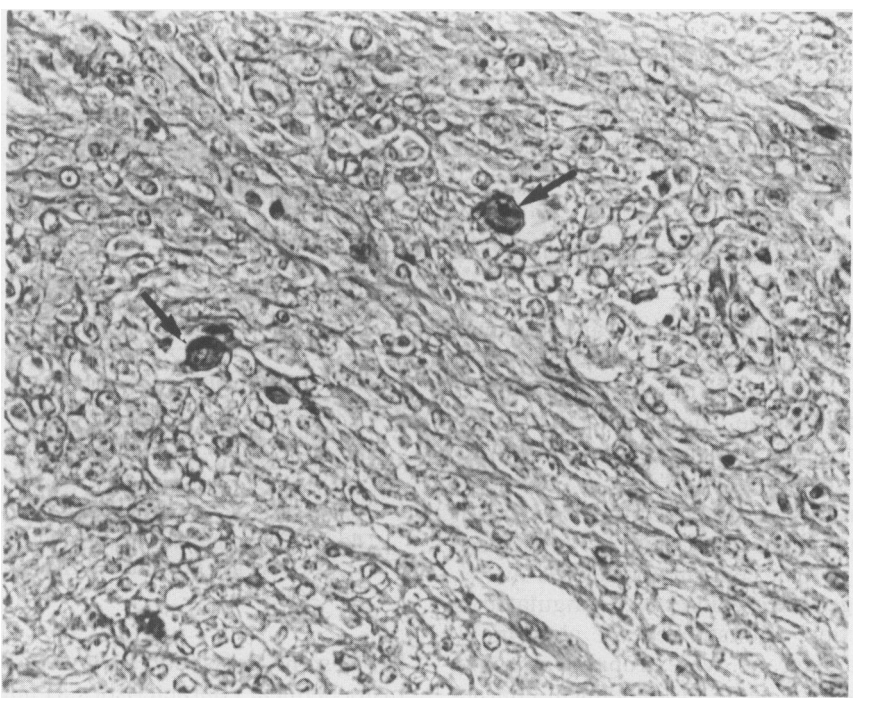


Fig. 8. Area of dense collagenous matrix 2 weeks after venom injection. There are very few regenerating fibres with abnormally small diameter (arrows). Trichrome × 1600.

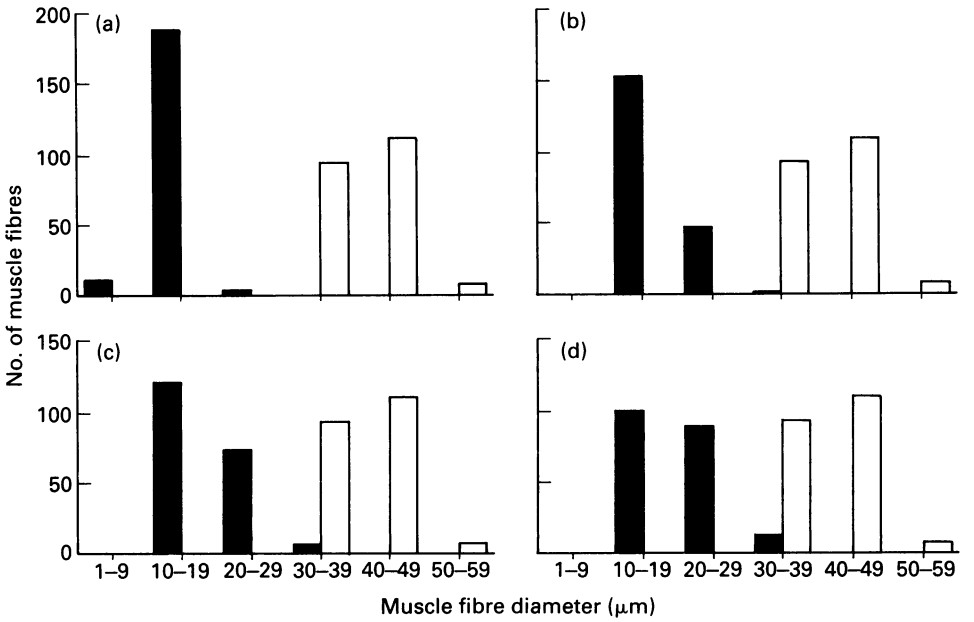


Fig. 9. Distribution of fibre diameters of regenerating muscle fibres at different time intervals after ■, injection of *Bothrops asper* venom. For comparison, □, the distribution of muscle fibre diameters in control skeletal muscle was included. a, One week; b, 2 weeks; c, 4 weeks; d, 8 weeks.

week after venom injection, together with very small, intact nerve bundles. These were located in closer proximity with respect to each other in comparison to nerve bundles in the control tissue.

Final stages in plausible muscle fibre atrophy (4 and 8 weeks)

Regenerating fibres reached an advanced stage of differentiation 1 month after venom injection, with necrotic regenerating fibres absent at this stage. The diameter of regenerating fibres did not increase significantly after 4 weeks (Fig. 9, Table 4). Very few regenerating fibres reached the size of adult fibres but they could still be recognized because of their centrally positioned nuclei. Other regenerating fibres were angulated in cross-section and showed an abnormally small diameter, i.e. were probably atrophic. These had the same histochemical characteristics as adult myofibres but presented a smaller diameter with centrally located nuclei. In some areas, fibres showing the same histochemical characteristics as adult fibres were surrounded by capillaries having an abnormal density and size.

Table 4. Diameter of regenerating muscle fibres after injection of *B. asper* venom

Time	Diameter (μm)*
Control†	39.1 ± 4.3^{a}
One week	12.9 ± 2.8^{b}
Two weeks	16.8 ± 3.6^{c}
Four weeks	18.3 ± 4.4^{d}
Eight weeks	18.8 ± 5.6^{d}

*Control values were obtained from uninjected mouse gastrocnemius muscle. Mean values with different superscripts (a, b, c, d) are significantly different ($P<0.01$) between them. Mean values of fibre diameter at 4 and 8 weeks do not differ significantly ($P>0.05$).

†Results are presented as mean ± s.d. ($n=200$).

Changes in hydroxyproline content of envenomated muscle

Hydroxyproline increased in muscle injected with venom. This increment was observed at 4 days, and reached values of 181 ± 18 and $180 \pm 12\%$ at 1 and 2 weeks, respectively, remaining elevated even 8 weeks after envenomation (Fig. 7).

Discussion

Previous studies had demonstrated that muscle regeneration is poor after myonecrosis induced by *B. asper* venom (Gutiérrez *et al.* 1984b, 1986), confirming clinical observations of many years. Gutiérrez *et al.* (1984b, 1986) proposed that the cause of this phenomenon was not myonecrosis itself, but damage to the microvasculature, since there is good regeneration after muscle damage induced by myotoxins which do not affect blood vessels (Harris & Maltin 1982; Gutiérrez *et al.* 1984b, 1989; Homsi-Brandeburgo *et al.* 1988).

Due to the significance played by vasculature in the process of muscle regeneration (Allbrook 1981; Plaghki 1985), and since capillaries are difficult to observe in routine histological preparations, it is necessary to study them using specific stains. However, since capillary endothelium might have different staining patterns to those observed for endothelium of larger vessels, it was important for the present study to find a general endothelial cell marker, commercially available for mouse skeletal muscle endothelial cells.

Our results indicate that both *Ulex europaeus* I lectin and antibody against Factor VIII-related antigen could not be used as general markers for murine endothelial cells. On the other hand, WGA proved to be a good general marker for endothelial cells in mouse skeletal muscle. In addition, the histochemical results obtained with this lectin incubated with different monosaccharides are in agreement with its reported biochemical specificity. However, it strongly stained cells other than endothelial cells in areas of muscle regeneration, such as those of the inflammatory infiltrate, particularly at 1, 2 and 3 days after venom injection, around the time when inflammation reached its peak. Enzymatic digestion with sialidase followed by WGA staining cleared the picture, since staining of inflammatory cells disappeared after this treatment, while retaining that of endothelial cells and fibroblasts. These two types of cell could be distinguished on the basis of their different morphological appearance.

After the onset of myonecrosis induced by *B. asper* venom, and the removal of necrotic material by phagocytic cells, there was a regenerative process which varied in different areas of the muscle. Interestingly, the early progression of muscle regeneration correlated with the integrity of the microvasculature. During the first week, there were areas of muscle with apparently normal regenerative fibres, corresponding to areas where the capillary network had not been drastically reduced. On the other hand, areas where the microvasculature was severely affected were characterized by impaired muscle regeneration. The finding of regenerating fibres showing signs of degeneration 4–6 days after venom injection is relevant. These 'degenerating-regenerative fibres' differed morphologically from muscle fibres affected directly by the venom. The former had an increased nuclear/cytoplasmic ratio and a vacuolated cytoplasm, lacked the clumped myofibrils found in fibres directly affected by venom myotoxins (Gutiérrez *et al.* 1990) and had reduced staining intensity with methyl green–pyronin. In contrast, necrotic muscle fibres that had been directly affected by the venom were not vacuolated and had clumped myofibrils and pycnotic nuclei. In addition, they lost desmin and glycogen early in the course of envenomation (Gutiérrez *et al.* 1990) and were not stained by methyl green–pyronin.

It is proposed that pathogenesis of muscle fibre damage in these two cases varies. In the case of fibres directly affected by the venom,

there is direct disruption of muscle plasma membrane by the action of myotoxins present in the venom (Gutiérrez *et al.* 1984a, b; Brenes *et al.* 1987). Thus, these fibres rapidly lose desmin, glycogen, nucleic acids, and their myofibrils are clumped into dense masses (Gutiérrez *et al.* 1984b, 1989). On the other hand, it is suggested that regenerating muscle fibres showing signs of degeneration are being affected by ischaemia, since these fibres were located in areas of reduced number of capillary vessels. Degenerative changes produced during ischaemia are not as drastic as in the case of direct membrane damage. Loss of myofibrillar proteins and glycogen takes more time and there is no prominent clumping of myofibrils.

Our data support the hypothesis of Gutiérrez *et al.* (1984b, 1986) who proposed that damage to microvasculature is the main cause of poor skeletal muscle regeneration after myonecrosis induced by *B. asper* venom. A similar observation of 'degenerating-regenerative fibres' has been described in older patients with Duchenne muscular dystrophy (Lipton 1979). Interestingly, this type of fibre was present in areas of extensive fibrosis and devoid of associated microvasculature (Lipton 1979).

Our findings indicate that *B. asper* venom affects not only the microvasculature, but also larger vessels such as intramuscular arteries and veins, inducing thrombosis and damage to the walls. It has been found, both experimentally and clinically, that a variety of crotaline snake venoms affect arteries inducing similar effects to those described in this work (Homma *et al.* 1967; Homma & Tu 1971; Queiroz & Petta 1984). Thus, if a major vessel is damaged, this will affect blood supply to a relatively large area of muscle tissue causing ischaemia and probably precluding regeneration. Therefore the combined effect of damage to microvessels and large vessels might be responsible for the lack of muscle regeneration and the consequent substitution of muscle fibres by fibrotic tissue in some areas.

Despite the finding of areas of apparently normal regenerative fibres during the first week after envenomation, most of these fibres had an abnormally reduced diameter at later time intervals. It was observed that *B. asper* venom drastically affected nerves. This effect might be due to a direct action of venom components on nerves, and/or a consequence of the ischaemia present in muscle tissue after haemorrhage and vasculature damage. Since innervation is a requisite for the completion of muscle regeneration (Allbrook 1981; Plaghki 1985), it is suggested that many regenerative fibres were denervated and consequently became atrophic. Previous studies have demonstrated that innervation is not required in the first stages of muscle regeneration (Hall-Craggs & Seyan 1975; Gulati 1988). This may explain why, despite the conspicuous nerve damage induced by the venom, there were many small regenerative fibres in samples taken at 6 and 7 days after envenomation. However, the diameter of these fibres increased only slightly after this time period reaching a plateau at 4 weeks, probably because innervation is required for the full differentiation of regenerating muscle fibres (Hall-Craggs & Seyan 1975; Harris *et al.* 1975; Gulati 1988).

There was evidence of nerve regeneration and revascularization at the time intervals where muscle regeneration took place. The regenerative features comprised very small and intact nerve bundles, with one or two axons, and small arteries and veins with an increased number of endothelial cells and a thickened wall. However, it is suggested that this regenerative process is insufficient to prevent necrosis of regenerative fibres and muscle fibre atrophy.

In conclusion, *B. asper* venom induces prominent muscle damage which is followed by a poor regenerative response. It is proposed that this venom affects muscle tissue in a complex way: (a) initially, there is a direct damage to muscle fibres, probably due to the action of myotoxins which disrupt the integrity of sarcolemma. Some of these myotoxins have been isolated and their mechanism of

action studied (Gutiérrez *et al.* 1984a, b; Brenes *et al.* 1987; Lomonte & Gutiérrez 1989). It is proposed that these toxins are responsible for the earliest pathological changes observed; (b) venom drastically affects the microvasculature inducing haemorrhage as well as thrombosis and damage to larger vessels. These effects induce an ischaemic condition which, in turn, affects regeneration of a population of muscle fibres during the first week after the onset of myonecrosis. Fibrosis further contributes to this condition; (c) by causing peripheral nerve damage, this venom induces denervation. It is proposed that this effect, together with a deficient reinnervation, is responsible for the large amount of apparently atrophic regenerative fibres observed even 2 months after envenomation.

Acknowledgements

The authors wish to thank Alberto Delgado, Shirley Quirós and Fernando Chaves for their excellent laboratory support. We are also indebted to Manuel Arce, Edgardo Moreno and Bruno Lomonte for critically reviewing the manuscript. This work was financed by Vicerrectoría de Investigación, Universidad de Costa Rica, research projects 741-88-022 and 801-87-108, and by CENDEISSS. J.M. Gutiérrez is recipient of a research career award from the Costa Rican National Scientific and Technological Research Council (CONICIT).

References

ALLBROOK D. (1981) Skeletal muscle regeneration. *Muscle Nerve* **4**, 234–245.

ALLEN H.J., JOHNSON E.A.Z. & MATTA K.L. (1977) A comparison of the binding specificities of lectins from *Ulex europaeus* and *Lotus tetragonolobus*. *Immunol. Comm.* **6**, 585–602.

ALLEN A.K., NEUBERGER A. & SHARON N. (1973) The purification, composition and specificity of wheat-germ agglutinin. *Biochem. J.* **131**, 155–162.

ARCE-ARENALES V. (1986) *A Comparative Histochemical Study of Bulbo-Urethral and Urethral Glands in Five Rodent Species.* PhD Thesis. University of Cambridge, UK.

BARWICK K.W. (1990a) S-100 protein. In *Atlas of Diagnostic Immunohistopathology*. Ed. L.D. True. Philadelphia: J.B. Lippincott. pp. 12.1–12.9.

BARWICK K.W. (1990b) Other intermediate filaments. In *Atlas of Diagnostic Immunohistopathology*. Ed. L.D. True. Philadelphia: J.B. Lippincott. pp. 4.1–4.10.

BHAVANANDAN V.P. & KATLIC A.W. (1979) The interaction of wheat germ agglutinin with sialoglycoproteins. The role of sialic acid. *J. Biol. Chem.* **254**, 4000–4008.

BOLAÑOS R. (1982) Las serpientes venenosas de Centroamérica y el problema del ofidismo. Primera parte. Aspectos zoológicos, epidemiológicos y biomédicos. *Revista Costarricense de Ciencias Médicas* **3**, 165–184.

BRENES F., GUTIÉRREZ J.M. & LOMONTE B. (1987) Immunohistochemical demonstration of the binding of *Bothrops asper* myotoxin to skeletal muscle sarcolemma. *Toxicon* **25**, 574–577.

BURGER M.M. & GOLDBERG A.R. (1967) Identification of a tumor-specific determinant on neoplastic cell surfaces. *Proc. Nat. Acad. Sci. USA* **57**, 359–366.

COOK H.C. (1982) Carbohydrates. In *Theory and Practice of Histological Techniques*. Second edition. Eds J.D. Bancroft & A. Stevens. Edinburgh: Churchill Livingstone, pp. 180–216.

GOLDSTEIN I.J. & HAYES C.E. (1978) The lectins; carbohydrate-binding proteins of plants and animals. *Adv. Carbohydr. Chem. Biochem.* **35**, 127–340.

GOLDSTEIN I.J., HOLLERMAN C.E. & SMITH E.E. (1965) Protein-carbohydrate interaction. II Inhibition studies on the interaction of concanavalin A with polysaccharides. *Biochemistry* **4**, 876–883.

GULATI A. (1988) Long-term retention of regenertive capability after denervation of skeletal muscle. *Anat. Rec.* **220**, 429–434.

GUTIÉRREZ J.M., ARCE V., BRENES F. & CHAVES F. (1990) Changes in myofibrillar components after skeletal muscle necrosis induced by a myotoxin isolated from the venom of the snake *Bothrops asper*. *Exp. Molec. Path.* **52**, 25–36.

GUTIÉRREZ J.M., CHAVES F., GENÉ J.A., LOMONTE B., CAMACHO Z. & SCHOSINSKY K. (1989) Myonecrosis induced in mice by a basic myotoxin isolated from the snake *Bothrops nummifer* (Jumping viper) from Costa Rica. *Toxicon* **27**, 735–745.

GUTIÉRREZ J.M., CHAVES F., MATA E. & CERDAS L. (1986) Skeletal muscle regeneration after myo-

necrosis induced by *Bothrops asper* (terciopelo) venom. *Toxicon* **24**, 223–231.

GUTIÉRREZ J.M., OWNBY C.L. & ODELL G.V. (1984a) Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*. *Exp. Molec . Path.* **40**, 367–379.

GUTIÉRREZ J.M., OWNBY C.L. & ODELL G.V. (1984b) Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake *Bothrops asper* (Fer-de-Lance) *Toxicon* **22**, 719–731.

HALL-CRAGGS E.C.B. & SEYAN H.S. (1975) Histochemical changes in innervated and denervated skeletal muscle fibres following treatment with bupivacaine (Marcain). *Exp. Neurol.* **46**, 345–354.

HARRIS J.B. & MALTIN C.A. (1982) Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus*. *Br. J. Pharmac.* **76**, 61–75.

HARRIS J.B., JOHNSON M.A. & KARLSSON E. (1975) Pathological responses of rat skeletal muscle to a single subcutaneous injection of a toxin isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. *Clin. Exp. Pharmacol. Physiol.* **2**, 383–404.

HOMMA M., KOSUGE T., OKONOGI T., HATTORI Z. & SAWAI Y. (1967) A histopathological study on arterial lesions caused by habu (*Trimeresurus flavoridis*) venom. *Japan. J. Exp. Med.* **37**, 323–335.

HOMMA M. & TU A.T. (1971) Morphology of local tissue damage in experimental snake envenomation. *Br. J. Exp. Path.* **52**, 538–542.

HOMSI-BRANDEBURGO M.I., QUEIROZ L.S., SANTO-NETO H., RODRIGUES-SIMIONI L. & GIGLIO J.R. (1988) Fractionation of *Bothrops jararacussu* snake venom: Partial chemical characterization and biological activity of bothropstoxin. *Toxicon* **26**, 615–627.

HOYER L.W., DE LOS SANTOS R.P. & HOYER J.R. (1973) Antihemophilic factor antigen. Localization in endothelial cells by immunofluorescent microscopy. *J. Clin. Invest.* **52**, 2737–2744.

LIPTON B. (1979) Skeletal muscle regeneration in muscular dystrophy. In *Muscle Regeneration*. Ed. A. Mauro. New York: Raven Press. pp. 31–40.

LOMONTE B. & GUTIÉRREZ J.M. (1989) A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **27**, 725–733.

LÓPEZ DE LEÓN A. & ROJKIND M. (1985) A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J. Histochem. Cytochem.* **33**, 737–743.

MONSIGNY M., ROCHE A.C., SENE G., MAGET-DANA R. & DELMOTTE F. (1980) Sugar-lectin interactions: How does wheat germ bind sialoglycoconjugates? *Eur. J. Biochem.* **104**, 147–153.

NONAKA I., TAKAGI A., ISHURA S., NAKASE H. & SUGITA H. (1983) Pathophysiology of muscle fibre necrosis induced by bupivacaine hydrochloride (Marcaine). *Acta Neuropathol.* **60**, 167–174.

OHSAKA A. (1979) Hemorrhagic, necrotizing and edema-forming effects of snake venoms. In *Snake Venoms*. Ed. C.Y. Lee. Berlin: Springer-Verlag. pp. 480–546.

PEARSE A.G.E. (1980) *Histochemistry Theoretical and Applied*. Fourth edition. Volume 1. London: Churchill Livingstone.

PEREIRA M.E.A., KISALUS E.C., GRUEZO R. & KABAT E.A. (1978) Immunochemical studies on the combining site of blood group H-specific lectin 1 from *Ulex europaeus* seeds. *Arch. Biochem. Biophys.* **185**, 108–114.

PETERS B.P., EBISU S., GOLDSTEIN I.J. & FLASHNER M. (1979) Interaction of wheat germ with sialic acid. *Biochemistry* **18**, 5505–5511.

PLAGHKI L. (1985) Régénération et myogenese du muscle strié. *J. Physiol., Paris* **80**, 51–110.

PROCKOP D.J. & UDENFRIEND S. (1960) A specific method for the analysis of hydroxyproline in tissues and urine. *Anal. Biochem.* **1**, 228–239.

QUEIROZ L.S. & PETTA C.A. (1984) Histopathological changes caused by venom of urutu snake (*Bothrops alternatus*) in mouse skeletal muscle. *Rev. Inst. Med. trop. Sao Paulo* **26**, 247–253.

SCHULTE B.A. & SPICER S.S. (1983a) Light microscopic detection of sugar residues in glycoconjugates of salivary glands and the pancreas with lectin-horseradish peroxidase conjugates. *Histochem J.* **15**, 1217–1238.

SCHULTE B.A. & SPICER S.S. (1983b) Light microscopic detection of sugar residues in secretory glycoproteins of rodent and human tracheal glands and the galactose-oxidase Schiff sequence. *J. Histochem. Cytochem.* **3**, 391–403.

STEVENS A. (1982) The haematoxylins. In *Theory and Practice of Histological Techniques*. Second edition. Eds J.D. Bancroft & A. Stevens. Edinburgh: Churchill Livingstone, pp. 109–121.

STEVENS A. & BANCROFT J.D. (1982) Proteins and nucleic acids. In *Theory and Practice of Histological Techniques*. Second edition. Eds J.D. Bancroft & A. Stevens. Edinburgh: Churchill Livingstone, pp. 145–154.

TU A.T. (1977) *Venoms: Chemistry and Molecular Biology*. New York: John Wiley.