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SHORT COMMUNICATION

IMMUNOCHEMICAL DETECTION OF PURIFIED CROTOXIN FROM *CROTALUS DURISSUS TERRIFICUS* VENOM IN THE MOTOR END PLATE OF STRIATED MUSCLE IN CBA/J MICE

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Electrophysiological studies of crotoxin, a potent neurotoxic fraction from *Crotalus durissus terrificus* venom, have shown a pre- and post-synaptic action. In order to determine the specific site of this activity, we performed an immunohistochemical analysis on striated muscle from CBA/J mice, injected with crotoxin (5 LD_{50}), *iv*, using a single-step immunoperoxidase assay with peroxidase-conjugated antibodies to whole venom. Control muscle and muscle obtained from treated animals 15 min after injection showed no staining. However, 30 min after injection, the neuromuscular motor end plate of mice who received crotoxin was clearly stained, including thin terminations, without any morphological alteration. Sixty min after administration, the motor end plate was no longer intact, but only roughly formed stained areas without clearly identifiable structures were present. These data show specific crotoxin binding to neural end plates in striated muscle, with a subsequent toxin-induced degeneration of this structure.

Key words: *Crotalus durissus terrificus*, crotoxin, snake venom, immunohistochemistry.

Snake bites continue to represent a health problem in Brazil and 13% of these accidents are caused by *Crotalus durissus terrificus* (1). The clinical picture of victims of *C. d. terrificus* bites is characterized by early signs of motor paralysis with mechanical respiratory distress (2) followed by muscular and renal involvement (3). The main toxic fraction of this venom, crotoxin, has two subunits, one with phospholipase A2 activity and the other, crotapotin, having no demonstrable enzymatic activity (4). Electrophysiological studies with crotoxin have shown the pre- and post-synaptic activity of this fraction (5,6), suggesting enzymatic degradation of the neural motor end plate. Until now, however, no morphological data have been presented to document this interpretation. In the present study, we investigated the morphological localization of crotoxin in striated muscle of mice.

Crotoxin was purified from *C. d. terrificus* crude venom by gel filtration on Sephadex G-75 (Pharmacia) followed by isoelectric pH precipitation (7). The Bradford method was used for protein determination and the purification was assessed by SDS-PAGE

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(8,9). This crotoxin was homogeneous and presented high toxicity, with an LD_{50} of 80 µg/kg, in mice.

Immunohistochemical detection was performed using peroxidase-conjugated antibodies prepared against whole venom. Anticrotalic horse serum was purchased from Instituto Butantan and extensively dialyzed against borate buffer to eliminate preservatives and other contaminants. Coupling with horseradish peroxidase was performed by the periodate method (10), using a 1:1 protein:peroxidase ratio. The efficiency of this conjugate was measured in a single-step ELISA assay (11) using crotoxin-coated microplates, resulting in a 1/400 titer in the conjugate. The *in vivo* assays were carried out by injecting groups of 3 CBA/J mice (18-22 g) with 5 µg of purified crotoxin in 0.1 ml PBS in the retroorbital plexus. This injection causes a 100% death rate at 3 h. Mice were killed by exposure to ether at 15, 30 and 60 min after injection. Clinical signs of toxicity, paralysis or respiratory distress appeared 15-30 min after crotoxin administration.

The hind leg muscle and other organs were carefully removed and immediately fixed by immersion in several changes of phosphate-buffered formalin to avoid pigment formation. Organs were embedded in Paraplast-plus^R resin and sections (4 µm) mounted on glue-coated slides. After deparaffinization and hydration, the sections were reacted with methanol-H_2O_2 to destroy endogenous peroxidase activity. Anti-*C. d. terrificus* conjugate was applied to the slides at 1/50 dilution in PBS and incubated for 1 h at 37°C in a humid chamber. After extensive washing, bound antibody was developed with diaminobenzidine-H_2O_2 under microscopic observation until a brown precipitate formed. The slides were counterstained with Mayer's hematoxylin and mounted with Permount^R. Representative fields were photographed with a Zeiss photomicroscope equipped with planapochromatic optics.

Examination of hind leg muscle routinely stained with hematoxylin-eosin disclosed no histological alterations in any animal, injected or control, with no myolysis or Zenker's degeneration. This fact can be ascribed to the short time after crotoxin administration which was insufficient to promote morphological alterations at this level.

Immunohistochemical analysis showed no stained material in muscle from control or injected mice 15 min after crotoxin administration. Muscle from 30-min injected mice presented clearly stained neural motor end plates, without any signs of morphological lesion (Figure 1A). The neural branches were clearly defined and small terminations were also seen. This finding corroborates the electrophysiological localization of crotoxin at the neuromuscular junction (5,6). Sixty min after injection, however, only roughly stained aggregates dispersed in the muscle, without clear definition of the stained structure, were observed (Figure 1B). This finding may be due to the enzymatic action of crotoxin on neuromuscular junctions, causing the loss of an identifiable structure, but we could not apply a counterstain specific for these junctions, due to the similar appearance of the immunohistochemical stain. A second problem was the scarcity of neuromuscular junction in thin sections.

The lung, spleen, liver and kidney of experimental mice showed no defined staining when the same immunohistochemical assay was used. This finding also corrobo-

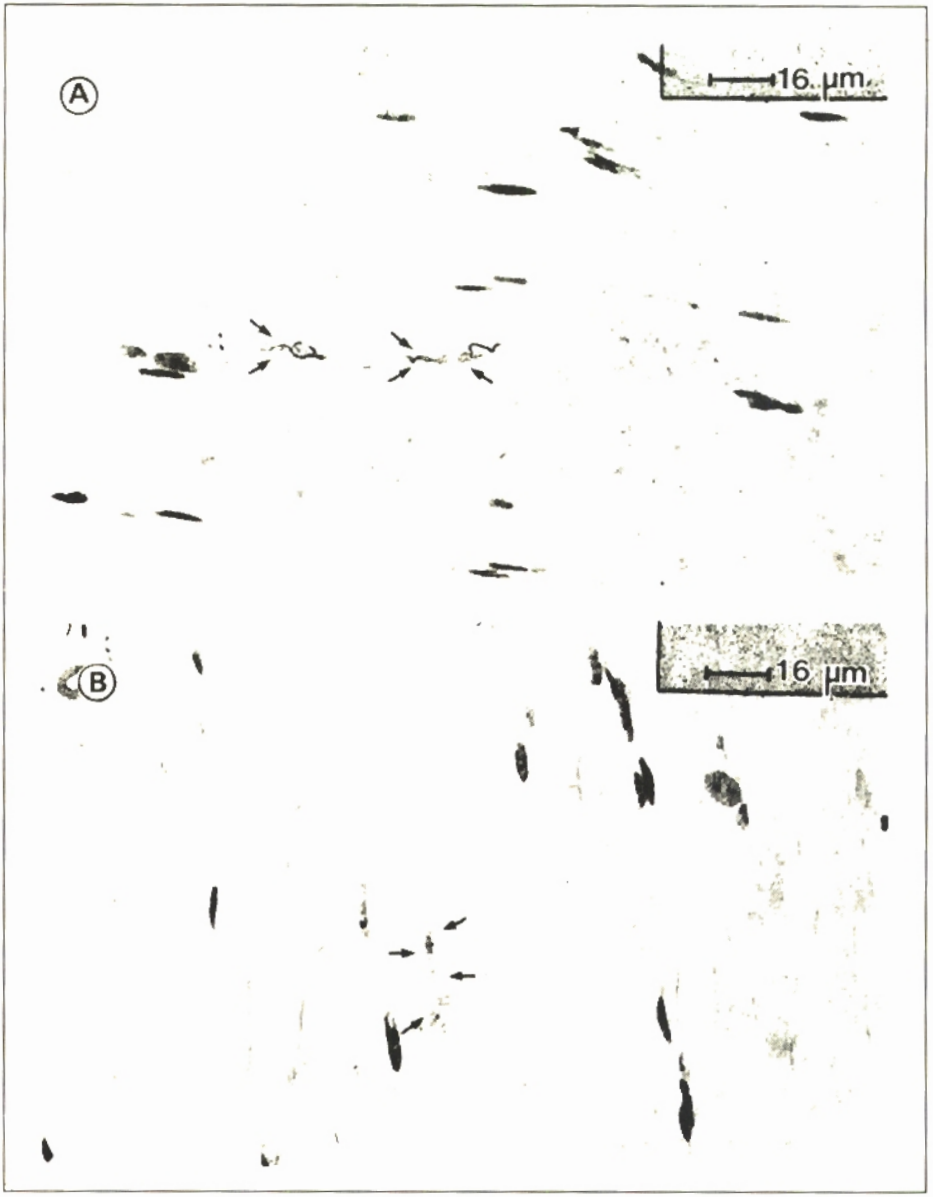

Figure 1 - Immunohistochemical detection of crotoxin in mouse hind leg muscle. The section, obtained after retroorbital administration of 5 µg purified crotoxin, was stained with peroxidase-conjugated antiserum to whole crotalic venom. *A*, Thirty min after crotoxin administration. Staining of neuromuscular motor end plate with intact structures having stained small terminations (arrows). *B*, Sixty min after crotoxin administration. The roughly stained masses are dispersed between muscular fibers (arrows).

rates the high specificity and concentration of crotoxin in the neuromuscular end plate of striated muscle. This specificity may be due to the crotapotin subunit, since it has been reported that, in *in vitro* regeneration studies with isolated subunits, crotapotin enhances the toxic activity of the venom, but inhibited the enzymatic activity of the complex toward a less specific substrate, such as the erythrocyte membrane (12). In future studies, we will use subunit-directed antibodies and isolated subunits to elucidate the specificity of this toxin at the morphological level.

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