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Pro-inflammatory activities in elapid snake venoms

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1 Snake venoms from the genera *Micrurus* (*M. ibiboboca* and *M. spixii*) and *Naja* (*N. naja*, *N. melanoleuca* and *N. nigricollis*) were analysed, using biological and immunochemical methods, to detect pro-inflammatory activities, cobra venom factor (COF), proteolytic enzymes, thrombin-like substances, haemorrhagic and oedema-producing substances.

2 The venoms of the five snake species activate the complement system (C) in normal human serum (NHS) in a dose-related fashion, at concentrations ranging from 5 μg to 200 μg ml^{-1} serum. Electrophoretic conversion of C3 was observed with all venoms in NHS containing normal concentrations of Ca^{2+} and Mg^{2+}, but only by venoms from *N. naja* and *N. melanoleuca* when Ca^{2+} was chelated by adding Mg^{2+}-EGTA.

3 Purified human C3 was electrophoretically converted, in the absence of other C components, by the venoms from *N. naja*, *N. nigricollis* and *M. ibiboboca*. However, only the venoms from *N. naja* and *N. melanoleuca* contained a 144 kDa protein revealed in Western blot with sera against COF or human C3.

4 All venoms, at minimum concentrations of 30 ng ml^{-1}, were capable of lysing sheep red blood cells, also in a dose-related fashion, when incubated with these cells in presence of egg yolk as a source of lecithin. Although the venoms from *M. spixii* and *N. nigricollis* showed detectable thrombin-like activity, these and the other venoms were free of proteolytic activity when fibrin, gelatin and casein, were used as substrates.

5 When tested on mice skin, all five venoms were capable of inducing an increase in vascular permeability and oedema, but were devoid of haemorrhagic producing substances (haemorrhagins).

6 These data provide evidence indicating that Elapidae venoms contain various pro-inflammatory factors which may be important in the spreading of neurotoxins throughout the tissues of the prey or human victim.

Keywords: Pro-inflammatory activities; snake venoms; complement activation; cobra venom factor; haemolytic activity; haemorrhagic; oedema and vascular increasing activities

Introduction

Venomous snakes belong to five families: elapidae, crotalidae, viperidae, hydrophidae and colubridae, and their venoms contain a vast number of substances with different biochemical and pharmacological activities. The venoms from some members of the Elapidae contain a 144 kDa glycoprotein, designated cobra venom factor (COF) (Müller-Eberhard & Fjellström, 1971). This protein displays some structural similarities and antigenic cross-reactivity with human C3 (Alper & Balavitch, 1976). There is evidence that COF is a physiological breakdown product of cobra C3 (Eggertsen *et al.*, 1983). COF generates active C3 convertase, COFBb by interacting with the C alternative components, factors B and D in the presence of Mg^{2+} (Götze & Müller-Eberhard, 1971; Götze, 1975). COFBb cleaves C3 into C3a and C3b (Shin *et al.*, 1969); C3a is released into the fluid phase while C3b binds to the bimolecular complex COFBb generating the trimolecular complex COFBbC3b, which now as a C5 convertase is able to cleave C5, the next component of the C cascade, into C5a and C5b (Smith *et al.*, 1982). Although the peptides C3a and C5a are mediators of the early events of the inflammatory process (Dias da Silva & Lepow, 1967; Dias da Silva *et al.*, 1967; Lepow *et al.*, 1967), C3b can be recognized by C receptors displayed on the phagocyte cell surface (Ross & Medof, 1985) and C5b serves as the starting for the C5b-C9 cytolytic complex organization (Pickering *et al.*, 1969).

Crotalidae venoms are abundant in proteases, some of which possess kinin-releasing activity (Rocha da Silva *et al.*, 1950; Geiger & Kortmann, 1977), others are capable of attacking fibrinogen catalyzing the release of fibrin peptides A and B from the terminal dissulphide knots of fibrinogen α and β chains (thrombin-like enzymes), or of splitting off fragments from the COOH terminals of α, β and γ chains of fibrinogen (fibrinogenolytic enzymes). Some may also act as fibrinolysins (plasminogen activating enzymes) (Stocker & Barlow, 1976; Stocker *et al.*, 1982), and others, present in venoms of viperidae, act on the vessel walls inducing haemorrhage (Mandelbaum & Assakura, 1988). Therefore, snake venoms are rich sources of enzyme-generating factors or enzymes capable of mediating, directly or indirectly, the early events of the inflammatory process. Inflammation at the site of the snake bite, together with the spreading action exerted by the hyaluronidase (Zeller, 1948), could facilitate the distribution of other toxic venom components, throughout the tissues of the prey.

The aims of the present work were to verify if the venoms of *Naja* and *Micrurus* contain substances endowed with pro-inflammatory activities as proteases, COF-like proteins, thrombin-like, haemolytic, C activators, haemorrhage inductors and mediators of vascular permeability.

Methods

Snake venoms

Venoms of *M. ibiboboca*, *M. spixii*, *N. naja*, *N. melanoleuca*, and *N. nigricollis* were provided by the Instituto Butantan,

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São Paulo, SP, Brazil, and the Bombain Institute, India. The venoms were extracted from 5–10 adult snakes using standard procedures, the samples from each species pooled, vacuum dried and stored at 4°C. Stock solutions were prepared in pH 7.2, 0.15 M phosphate buffered-saline (PBS) at 1.0 mg ml^{-1}. Cobra venom factor (COF) from *N. naja* venom was purchased from Diamedix FL, U.S.A.

Sera and anti-sera

Normal human serum (NHS) was obtained from healthy donors; rabbit anti-COF was kindly provided by Dr Ramalho-Pinto, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, MG, Brazil; goat anti-human C3, (Atlantic Antibodies, Scarborough, ME, U.S.A.); peroxidase goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, U.S.A.) and rabbit anti-goat IgG (Kirkegaard and Perry Lab. Inc., Gaithersburg, MA, U.S.A.).

Activation of C

Samples of 200 µl of normal human serum (NHS) were treated with 1.0, 5.0, 15.0 and 40.0 µg of snake venoms previously dissolved in PBS, or with PBS alone (control samples), for 1 h at 37°C and stored at − 20°C until assay.

Haemolytic C-assay

Volumes of NHS pretreated as described above, diluted 1:30 in pH 7.4 triethanolamine-saline buffer containing 0.1% gelatin, 15 mM $CaCl_2$ and 5 mM $MgCl_2$ (TBS^{2+}). Volumes of NHS ranged from 50 µl to 450 µl in 50 µl increments, and the volumes were adjusted to a final volume of 450 µl with TBS^{2+}. Two control tubes each containing 450 µl TBS^{2+} (cell blank) or distilled water (100% lysis) were always included. Three hundred microliters of EA, [sheep erythrocytes (E) optimally sensitized with rabbit antibodies (A) against E] at 5×10^8 ml^{-1} in TBS^{2+} were added to each tube. The mixtures were incubated for 1 h at 37°C; the reaction was then stopped by the addition of 2.0 ml cold saline to all tubes, except in the 100% lysis control to which 2.0 ml water was added. Tubes were centrifuged at 1300 r.p.m. for 10 min and the haemoglobin released into the supernatants determined spectrophotometrically at 412 µm, and the number of haemolytic sites (Z) calculated. The number of haemolytically active sites of each C component can be represented by $Z = -\ln (1-Y)$, the negative natural logarithm of the number of erythrocytes not lysed. For 63% haemolysis, $Z = 1$, which corresponds to one haemolytically active site per erythrocyte.

SDS-polyacrylamide gel electrophoresis (SDS–PAGE), Western blot (WB), double immunodiffusion (DID) and immunoelectrophoresis (IE)

SDS–PAGE and WB Whole snake venoms are analysed in 10% polyacrylamide slab gel using a Protean II (Bio-Rad Laboratories, Richmond, CA, U.S.A.). All samples (25 µl) were used at concentrations of 1 mg ml^{-1}. Some gels were silver stained by conventional methods to analyse the electrophoretic patterns of the snake venoms, while others were used for transblotting the electrophoretically separated protein bands onto nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Richmond, St. Louis, MO, U.S.A.). After transblotting, the nitrocellulose membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS), incubated for 1 h at room temperature with rabbit anti-COF (diluted 1:2000) or with goat anti-human C3 (diluted 1:100). After thorough washing with TBS, the membranes were incubated with the peroxidase conjugated anti-rabbit IgG or anti-goat IgG, diluted 1:3000, and the affinity staining for horseradish peroxidase was developed with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO, U.S.A.).

NHS or purified human C3 pretreated or untreated with snake venoms were analysed by IE according to described method (Grabar & Burtin, 1964), the C3, and its cleavage products were revealed with goat IgG anti-C3. DID analyses were performed as described by Crowle (1961).

Analysis of proteolytic activity

The proteolytic activity of the snake venoms was tested either using gelatin incorporated in SDS–PAGE gel (zymogram method) or casein in solution (caseinolytic method), according to described methods (Heussen & Dowdle, 1980; Lomonte & Gutiérrez, 1983). Thrombin-like and fibrinolytic activities were tested by the method of Baughman (1970).

Determination of indirect haemolytic activity

Increasing amounts of venoms starting from 30.0 ng and increasing to 300.0 ng (30.0 ng increments), in a volume of 100 µl were added to a row of test tubes. To 100 µl of egg yolk solution (300 µl of 10% egg yolk solution in saline plus 250 µl of 0.001 M $CaCl_2$), and 200 µl of sheep red blood cells (E) at 1.5×10^8 in saline were added to each tube. Three control tubes each containing 200 µl saline and 200 µl E (cell blank, egg yolk-control, and 100% lysis) were always included. After incubation for 1 h at 37°C, the reaction was stopped by adding 2.0 ml of cold saline to all tubes, except for the 100% lysis tube to which was added 2.0 ml water. The tubes were centrifuged for 10 min and the haemoglobin released in the supernatants determined spectrophotometrically at 412 nm, and the number of Z calculated.

Haemorrhagic (HPS), oedema and increasing vascular permeability (IVP) producing substances

The presence of HPS in the snake venoms was evaluated as described by Kondo *et al.* (1960). EPS and IVP producing substances in the venoms were determined according to the procedures of Yamakawa *et al.* (1976) with some modifications. Briefly, hind paw oedema was produced by a subplantar injection of 50 µl venom solution (100 µg ml^{-1}) or an equal volume of PBS into the right and left hind-paw of outbred mice, respectively. After different periods of time, the animals were killed according to ethical procedures and both feet were cut at the proximal joint and weighted in analytical balance. Oedema was expressed as the percentage increase in weight of the envenomed foot relative to the weight of the saline-injected foot.

Ten minutes before the animals were killed, mice were injected intravenously with a 80 µl solution of Evans blue dye 2.5% (w/v) in PBS. The feet used for determination of oedema were cut in small pieces incubated with formamide and the quantity of extracted material was determined from an Evan's blue dye standard curve (Garcia Leme & Wilhelm, 1975). Capillary permeability increasing activity was expressed as the increase (µl ml^{-1}) in the dye-complexed albumin of the envenomed foot relative to the dye complexed albumin of the saline-injected foot.

Results

C-activation

Figure 1 shows that all five snake venoms used in this work, were able to activate C, in a dose-related fashion, and under conditions in which the classical and alternative pathways of C activation were allowed to function. At a concentration of 250 µg ml^{-1} the C consumption induced by all venoms was near 100% (Figure 1). This C activation was followed by a conversion of the C3 electrophoretic mobility by the venoms of *N. naja* and *N. melanoleuca* (total conversion), *N. nigricollis*, *M. ibiboboca* and *M. spixii* (partial conversion) (Figure 2). When, however, Mg^{2+}-EGTA was added into the incuba-

tion mixtures at final concentrations of 1 mM $MgCl_2$ plus 10 mM EGTA, only the venom of *N. naja* and *N. melanoleuca* retained the ability to convert C3 (data not shown). Purified human C3 was electrophoretically converted, in the absence of other C components, by the venoms from *N. naja*, *N. nigricollis* and *M. ibiboboca* (Figure 3).

Presence of COF-like and C3-like proteins in the snake venoms

A COF-like or C3-like 144 kDa protein was clearly detected in the venoms of *N. melanoleuca* and *N. naja* but not in the venoms of the other species, in immunoblots using anti-COF or anti-human C3 (Figure 4). These proteins showed complete immunological identity with purified COF in double immunodiffusion analysis (data not shown).

Proteolytic, thrombin-like and fibrinolytic activities

Proteolytic activities assayed either using gelatin dispersed in SDS-polyacrilamide gels or casein and fibrinogen in solution, were not detected in any of the five venoms studied. When, however, the venoms were tested for their capacity to induce bovine fibrinogen coagulation, the venoms of *M. spixii* and *N. nigricollis* presented a small but clear thrombin-like activity (Table 1).

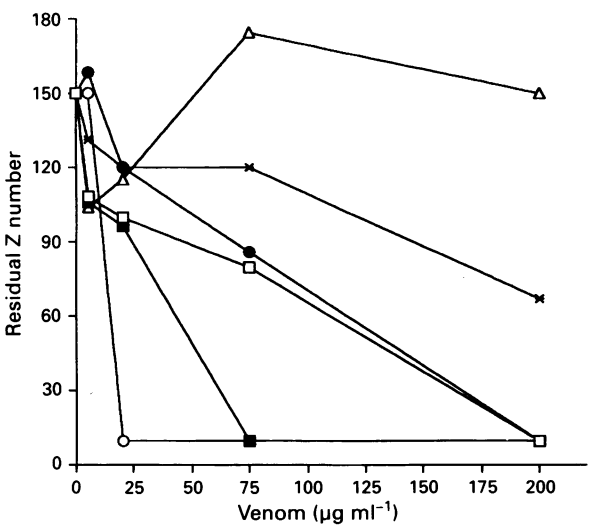

Figure 1 Consumption of complement in normal human serum by different amounts of elapid venoms using *C. d. terrificus* (△), *N. naja* (●), *N. nigricollis* (×), *N. melanoleuca* (●), *M. ibiboboca* (■) and *M. spixii* (□).

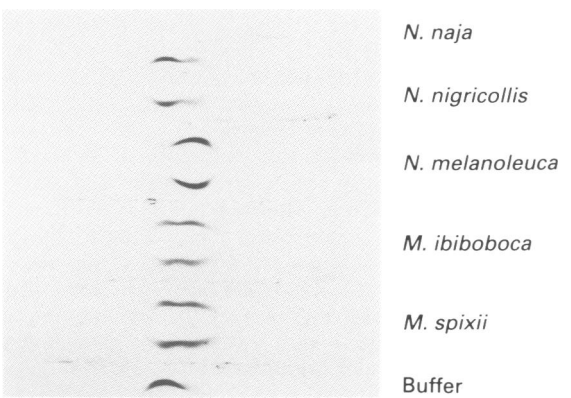

Figure 2 C3 conversion by elapid venoms. Immunoelectrophoresis analysis of normal human serum treated with the following venoms: *N. naja* (a), *N. nigricollis* (b), *N. melanoleuca* (c), *M. ibiboboca* (d) and *M. spixii* (e) on C3 in serum.

Haemorrhagic, oedema-inducing and increasing vascular permeability activities

The elapid venoms were able to increase the vascular permeability and oedema (Figure 5a,b). These venoms were devoid of haemorrhagic inducing activity (data not shown).

Haemolytic activity

All five venoms were able to induce lysis of sheep red blood cells, in a dose-related fashion, when incubated with these cells in the presence of egg yolk as a source of lecithin (Figure 6).

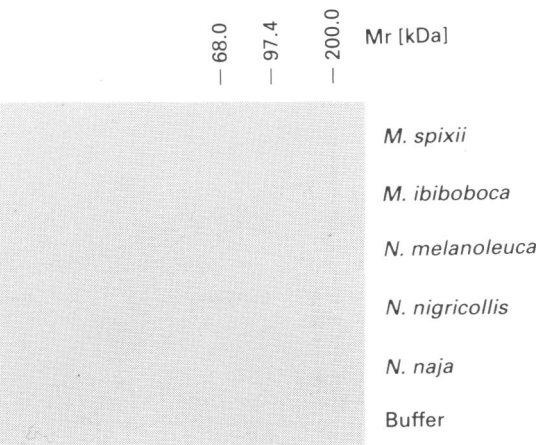

Figure 3 Proteolytic action of the elapid venoms on purified C3 Western blot analysis of purified human C3 samples treated with the venoms from *M. spixii* (a), *M. ibiboboca* (b), *N. melanoleuca* (c), *N. nigricollis* (d), *N. naja* (e) or with buffer (f) and developed with anti-human C3 antibodies.

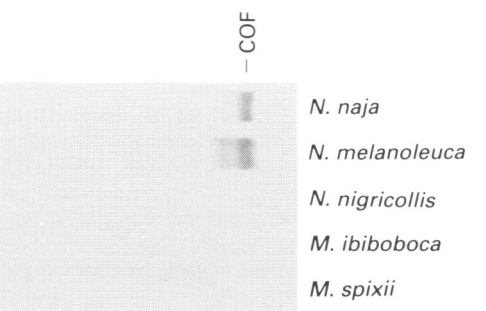

Figure 4 Identification of COF-like proteins in elapid venoms. Western blot analysis of venoms from *N. naja* (a), *N. melanoleuca* (b), *N. nigricollis* (c), *M. ibiboboca* (d), *M. spixii* (e) developed with anti-COF.

Table 1 Thrombin-like activity of *micrurus* and *naja* venoms

Venoms	*Thrombin-like activity* 500 μg ml^{-1}	1000 μg ml^{-1}
M. ibiboboca	–	–
M. spixii	60 min*	25 min*
N. naja	–	–
N. nigricollis	200 min*	75 min*
N. melanoleuca	–	–

– No clot
*Fibrinogen coagulation time
Samples of purified bovine fibrinogen in buffer containing Ca^{2+} were mixed with equal volume of venom solutions containing 5 μg and allowed to clot at room temperature. Clotting time was recorded.

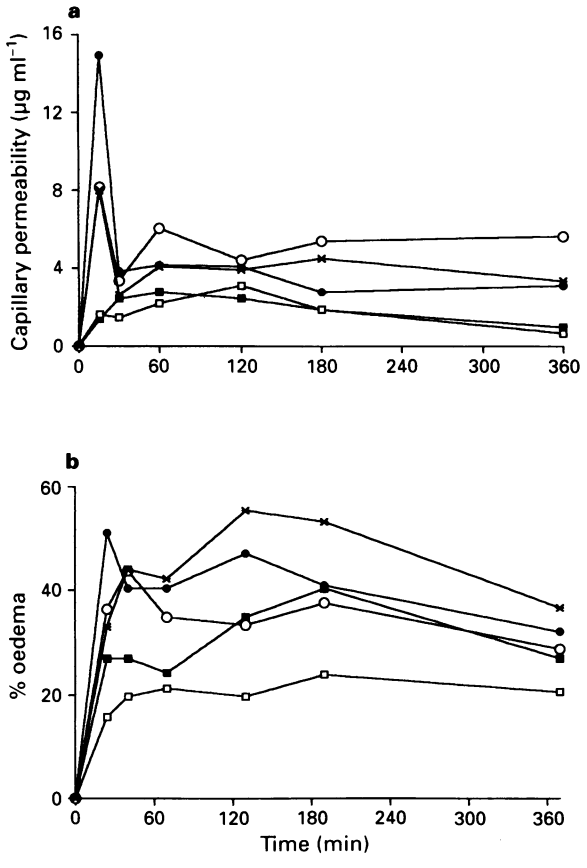

Figure 5 Time-course of the increasing vascular permeability (a) and oedema forming activity (b) by injection of 5 μg of elapidic venom into hind paw of mice. *N. naja* (●), *N. nigricollis* (×), *N. melanoleuca* (○), *M. ibiboboca* (□) and *M. spixii* (■).

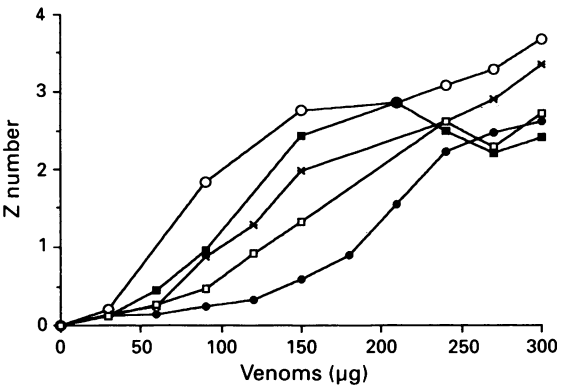

Figure 6 Haemolytic activity present in elapid venoms. Increasing amounts of venoms (30.0–300.0 ng), were added to a series of tubes containing egg yolk and sheep red blood cells. Haemolysis was spectrophotometrically determined and the number of Z calculated. *N. naja* (●), *N. nigricollis* (×), *N. melanoleuca* (○), *M. ibiboboca* (□) and *M. spixii* (■).

Discussion

The toxins present in snake venoms, used by snakes for effective immobilization of prey and for protection against predators, must rapidly reach the target organs. Therefore, mechanisms facilitating the distribution of the toxins throughout the tissues of the bitten animal must be activated. Hyaluronidase, which is present in almost all venoms, was the first of such mechanisms to be described (Zeller, 1948)., Another mechanism which could fulfil this spreading function is local acute inflammation at the site of bite or injection of the venoms. Some potentially pro-inflammatory factors such as proteolytic enzymes and activators of clotting, C and kallicrein-kinin systems have been detected in Crotalidae and Viperidae venoms. The action of these factors on the corresponding host substrates may release endogenous mediators of inflammation (Rocha e Silva *et al.*, 1950; Müller-Eberhard & Fjellström, 1971; Stocker & Barlow, 1976; Geiger & Kortmann, 1977; Stocker *et al.*, 1982).

The elapid venoms analysed contain components able to activate the C system. Consumption of haemolytic activity and conversion of C3 into products with electrophoretic mobility distinct from the native C3 molecule were observed in samples of human serum treated with these venoms. Some, such as *N. naja* and *N. melanoleuca* venoms which contain COF activate C by forming a COF-B complex, a C3-like convertase (Götze, 1975). *N. naja*, *N. nigricollis* and *M. ibiboboca* venoms cleave human purified C3 in the absence of other C factors. This effect could be attributed to some proteolytic enzymes present in these venoms. *M. spixii* venom, which is free of COF and is unable to cleave C3 directly, may enter into the C cascade through a Ca^{2+}-dependent mechanism. The C-activators certainly contribute to the local lesions induced by venoms, through the release into the injured tissues of the anaphylatoxins C3a and C5a. These two peptides are known to be mediators of the early events of the acute inflammation by increasing local vascular permeability, contracting smooth muscle, releasing histamine from mast cells, and by attracting leukocytes (Dias da Silva & Lepow, 1967; Dias da Silva *et al.*, 1967; Lepow *et al.*, 1967).

The thrombin-like enzymes detected in some venoms such as *M. spixii* and *N. nigricollis* by converting *in vitro* fibrinogen into a fibrin clot, may alter the local microcirculation through precipitous formation of thrombin. Formation of small thrombi could disturb the circulation (Stocker *et al.*, 1982).

The haemolytic inducing-activity present in all elapid venoms tested in this work can be attributed to their phospholipase A_2 contents since haemolysis only occurs when venom, sheep erythrocytes, and a lecithin as source of lysolecithin were mixed simultaneously. In animal or human tissues where snake venoms had previously been injected, phospholipase A_2 can degrade the cell membrane phospholipids into arachidonic acid. This compound can be metabolized by the enzyme cyclo-oxygenase to prostaglandins and by the enzyme lipoxygenase to hydroxy-eicosatetraenoic acids (HETEs) and derivatives of 5-hydroperoxy-eicosatetraenoic acid termed leukotrienes (Lewis & Austen, 1988). According to the type of isomerase enzymes present in the affected tissues, distinct cyclo-oxygenase products will result including prostaglandins, thromboxane, or prostacyclins. Some are vasoconstrictors (thromboxanes), others are vasodilators (prostacyclins), while others can increase vascular permeability (prostaglandins E_2).

The proteolytic enzymes present in these venoms could be responsible for the bradykinin formation and for breakdown of some clotting or C components.

The synergistic effect of the mediators of inflammation released by the action of proteolytic enzymes and activators of C, clotting and kallikrein-kinin systems or resulting from degradation of cell membrane phospholipids, by phospholipase A_2, may facilitate a rapid passage of the neurotoxins from the tissues to the blood. The observed oedema surrounding the sites of injection of the venoms, reinforces the suggestion that the *in vitro* formation of mediators, may also occur *in vivo* under conditions allowing their action.

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