Toxicon, Vol. 25, No. 9, pp. 947–955, 1987.
Printed in Great Britain.

0041–0101/87 $3.00+ .00

DETECTION OF PROTEINS ANTIGENICALLY RELATED TO *BOTHROPS ASPER* MYOTOXIN IN CROTALINE SNAKE VENOMS

BRUNO LOMONTE[1], EDGARDO MORENO[2,3] and JOSÉ MARÍA GUTIÉRREZ[1]*

[1]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica,
[2]Centro de Investigación en Biología Celular y Molecular, Universidad de Costa Rica, San José, and
[3]Universidad Nacional, Heredia, Costa Rica

(*Accepted for publication* 5 *March* 1987)

B. LOMONTE, E. MORENO and J. M. GUTIÉRREZ. Detection of proteins antigenically related to *Bothrops asper* myotoxin in crotaline snake venoms. *Toxicon* 25, 947–955, 1987. — The presence of components antigenically related to *Bothrops asper* myotoxin was investigated by Western blotting and immunoelectrophoretic techniques. *B. asper* myotoxin is a non-glycosylated monomeric phospholipase A with a molecular weight by SDS-PAGE of 16,000 and isoelectric point of pH 9.8–10.0. Results showed that proteins in the venoms of *B. nummifer, B. godmani, B. schlegelii, B. picadoi*, and *Agkistrodon bilineatus* were recognized by monospecific antibodies to *B. asper* myotoxin raised in rabbit and sheep. Western blotting indicated that cross-reacting proteins have a molecular weight of 16,000, with the exception of that of *B. picadoi*, which is of 24,000 mol. wt. However, immunoelectrophoresis indicated that these components are highly heterogeneous in charge, ranging from basic to acidic proteins. The cross-reacting component(s) present in newborn *B. asper* venom has a different charge from that of the 'adult-type'. Venoms from newborn specimens showed an additional cross-reacting band of 18,000 mol. wt. Myotoxin is an abundant component in adult *B. asper* venom. Myotoxin–antimyotoxin complexes had different electrophoretic mobilities in rocket immunoelectrophoresis depending upon the species in which monospecific immune sera were produced.

INTRODUCTION

LOCAL myonecrosis is one of the consequences of poisoning by snakes of the subfamily Crotalinae (OWNBY, 1982). Myotoxic activity has been demonstrated in the venoms of several species of the genera *Bothrops, Crotalus* and *Lachesis* from Costa Rica (GUTIERREZ and CHAVES, 1980). A myotoxin which was isolated from the venom of *B. asper* (GUTIERREZ *et al.*, 1984) belongs to the group of myotoxic phospholipases A_2 (GUTIERREZ *et al.*, 1984; GUTIERREZ and CERDAS, 1984). By neutralization experiments, it was shown that the myotoxin is the main component responsible for the myotoxic activity of crude venom (LOMONTE *et al.*, 1985). In previous work (LOMONTE *et al.*, 1985), the presence of components antigenically related to *B. asper* myotoxin in other crotaline venoms from Costa Rica was investigated. A cross-reaction with the venom of *B. schlegelii* was found. We have further investigated the presence of serologically related components in other snake venoms using more sensitive immunochemical techniques. In this work, we describe the occurrence and some biochemical characteristics of proteins related to *B. asper* myotoxin from the venoms of five crotaline species.

*To whom reprint requests should be addressed.

MATERIALS AND METHODS

Isolation and characterization of B. asper *myotoxin*

Myotoxin was isolated from *B. asper* venom by two cycles of ion-exchange chromatography on CM-Sephadex C-25, as described by GUTIERREZ *et al.* (1986*a*). Homogeneity was evaluated by disc-polyacrylamide gel electrophoresis at pH 4.3 (REISFELD *et al.*, 1962). Molecular weight was estimated by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE), according to LAEMMLI (1970). Isoelectric point was determined by analytical isoelectric-focusing on polyacrylamide gel using ampholytes from pH 3 to 10, according to the Instruction Manual of Pharmacia (Pharmacia Fine Chemicals Co., Uppsala). The presence of carbohydrates was studied by periodic acid Schiff staining and concanavalin A–peroxidase conjugate as described by RAMIREZ *et al.* (1983).

Anti-myotoxin antisera and polyvalent antivenom

Antisera against purified *B. asper* myotoxin were produced in rabbits and sheep. Initial doses (2 mg) were given in Freund's complete adjuvant (Difco Laboratories, Detroit) by i.m. route (HERBERT, 1978). Booster injections were performed with 1 mg doses dissolved in sodium alginate and administered by the s.c. route every two weeks during a period of two months. The antibody response was assessed by an immunodiffusion test against *B. asper* venom (10 mg/ml) and myotoxin (2 mg/ml). Specificity was assessed by immunoelectrophoresis against venom and myotoxin (OUCHTERLONY and NILSSON, 1978). Sheep and rabbit antisera were used in gel precipitation techniques, whereas rabbit antiserum was used in Western blotting techniques. Polyvalent antivenom was produced in horses immunized with a mixture of equal parts of the venoms of *Bothrops asper*, *Crotalus durissus* and *Lachesis muta* (BOLAÑOS and CERDAS, 1980).

Preparation of conjugates

The IgG fraction of rabbit anti-myotoxin serum was isolated by affinity chromatography on protein A-Sepharose 4B (Pharmacia) (LANGONE, 1982). Protein A was obtained from supernatants of cultures of methicillin-resistant *Staphylococcus aureus* Cowan I (strain A676), as described by RAMIREZ *et al.* (1983). IgG was conjugated to horseradish peroxidase (HRP type IV; Sigma Chemical Co., St Louis) according to the method of NAKANE and PIERCE (1966). Conjugates were diluted 1:2 in glycerol, and stored at 4°C.

Titration of the anti-myotoxin IgG–HRP conjugate was performed by dot blotting (TOWBIN and GORDON, 1984). Drops of 2 μl of serial dilutions of *B. asper* venom, ranging from 4 to 250 ng, were adsorbed onto nitrocellulose paper strips (Bio-Rad Laboratories, Richmond). After 5 min, unbound sites on the strips were blocked with 1% bovine serum albumin (Sigma), 1% casein (BDH Chemicals, Poole), 0.025 M barbital solution (pH 8.6) for 6 hr. Then, different amounts of conjugate in 0.1% albumin, 0.1% casein barbital buffer were added to each strip, to give final dilutions of 1:50, 1:100, 1:250, 1:500 and 1:1000. Incubation was carried out at 4°C for 16 hr. After four washings (15 min each) with the same solution, enzymatic activity was detected using 4-chloro-1-naphtol (Bio-Rad) and hydrogen peroxide as substrate, according to the manufacturer's instructions (Bulletin #1141, Bio-Rad).

Western blotting and dot-blotting analyses

Different *B. asper* venom samples were analysed by Western blotting (BERS and GARFIN, 1985). Samples of 40 μg of each venom dissolved in distilled water were reduced with 2-mercaptoethanol and separated by 15% SDS-PAGE. A control of purified *B. asper* myotoxin was included. After separation, proteins in the gel were transferred electrophoretically to nitrocellulose paper at 70 mA for 16 hr according to the method of TOWBIN *et al.* (1979). Then, nitrocellulose was coated as described, and myotoxin was detected with rabbit anti-myotoxin antibodies followed by HRP-labelled protein A. In all cases, samples were tested for the presence of endogenous peroxidase activity.

The venoms of *B. asper, B. schlegelii, B. nummifer, B. picadoi, B. godmani, B. lateralis, B. ophryomegas, B. nasutus, B. lateralis, Lachesis muta stenophrys* and *Crotalus durissus durissus* from Costa Rica, and *Agkistrodon bilineatus* from Guatemala, were analysed by Western blotting, as described above. All venom samples were tested by dot blotting in native conditions against anti-myotoxin HRP-conjugated rabbit IgG.

Two-dimensional SDS-PAGE

In order to investigate the possibility of formation of myotoxin aggregates during electrophoresis, an excess of *B. asper* venom (1 mg) was reduced with 2-mercaptoethanol, loaded on a 12% gel, and separated in the first dimension. Then, the corresponding polyacrylamide strip was cut, re-boiled in the presence of 2-mercaptoethanol, and placed horizontally on a second 12% SDS-PAGE slab. A control *B. asper* venom was included in a separate well in the same gel. After the second dimension, proteins were transferred to nitrocellulose, and myotoxin was detected as described, using HRP-conjugated antibodies. Protein staining of the gel was performed with Coomassie Brilliant Blue R-250.

Immunoelectrophoretic analyses

Venoms which reacted with antibodies to *B. asper* myotoxin in Western blotting tests were analysed by immunoelectrophoresis (OUCHTERLONY and NILSSON, 1978) against sheep anti-myotoxin serum. Samples of 75 μg/15 μl of each venom were separated electrophoretically on 1% agarose gels in 0.025 M barbital buffer, pH 8.2, at 20 mA during 2 hr. Diffusion against sheep serum was allowed for 24 hr at room temperature, and then the plates were washed with phosphate-buffered saline solution, pH 7.2, and stained with Amidoblack 10 B.

Rocket immunoelectrophoresis (WEEKE, 1973) of the venoms was performed on 1% agarose gels, pH 8.2, containing either 5% v/v rabbit antiserum or sheep antiserum to myotoxin. Samples of 50 μg/10 μl of each venom were run at 12 mA for 4 hr. Plates were washed and stained as described.

Crude *B. asper* venom was analysed by crossed immunoelectrophoresis (WEEKE, 1973) against sheep antiserum to myotoxin. A sample of 50 μg/10 μl was separated in the first dimension at 20 mA for 2 hr. The second dimension was run in 5% v/v sheep antiserum-containing agarose, at 12 mA for 5 hr.

RESULTS

Properties of myotoxin

Purified *B. asper* myotoxin appeared as a single band in disc-polyacrylamide gel electrophoresis at pH 4.3, and as a single band of 16,000 mol. wt in SDS-PAGE either in the absence or in the presence of 2-mercaptoethanol as reducing agent (not shown). The toxin focused at a single band, pH 9.8 – 10.0 in analytical polyacrylamide isoelectric focusing. Neither the periodic acid Schiff stain nor the concanavalin A – peroxidase

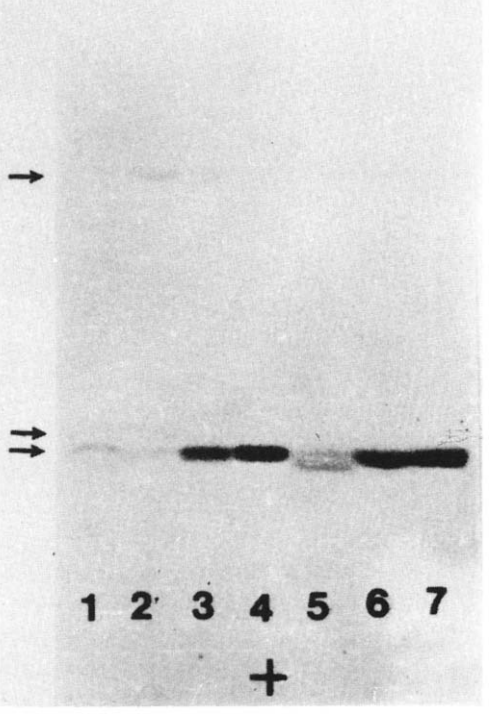

FIG. 1. IMMUNODETECTION OF MYOTOXIN AND CROSS-REACTING PROTEINS FROM DIFFERENT *Bothrops* VENOMS.

Venoms were separated in SDS-PAGE, electrophoretically transferred to nitrocellulose paper, and immunoenzymatically detected with a conjugate of anti-myotoxin rabbit IgG – peroxidase and 3, 3-diaminobenzidine as precipitable substrate. Fifty micrograms of newborn *B. asper* venom from the Pacific region (1); 50 μg of newborn *B. asper* venom from the Atlantic region (2); 50 μg of adult *B. asper* venom from Honduras (3); 10 μg of purified *B. asper* myotoxin (4); 50 μg of *B. schlegelii* venom (5); 50 μg of adult *B. asper* venom from the Pacific region (6); and 50 μg of adult *B. asper* venom from the Atlantic region (7). The arrows indicate faint protein bands. Notice the two bands detected in the newborn *B. asper* venoms and in *B. schlegelii* venom.

(RAMIREZ *et al.*, 1983) method detected carbohydrates in the electrophoretically-separated myotoxin.

Antisera to myotoxin

Both rabbit and sheep antisera formed two superimposed parallel precipitation arcs by immunoelectrophoresis against both crude *B. asper* venom and purified myotoxin.

Western blotting and dot blotting analyses

The working dilution of HRP – anti-myotoxin conjugate selected by dot blotting titration was 1:100. This dilution detected the myotoxin present in 8 ng of *B. asper* venom. By Western blotting, venoms of *B. asper* from the Atlantic and Pacific regions of Costa Rica showed one band of molecular weight 16,000, with identical mobility to the purified myotoxin (Fig. 1). Myotoxin was also detected in *B. asper* venom from Honduras (Fig. 1). Less intense reactions corresponding to molecular weights of 16,000 and 18,000 were observed with both Atlantic and Pacific *B. asper* venom pools obtained from specimens of less than 30 days of age (Fig. 1). The reaction with venoms from newborn specimens was slightly stronger in that of the Pacific than in that of the Atlantic. In some instances, a faint band of 65,000 mol. wt was observed in *B.* asper venoms (Fig. 1), but this reaction was not reproducible in all immunotransferences.

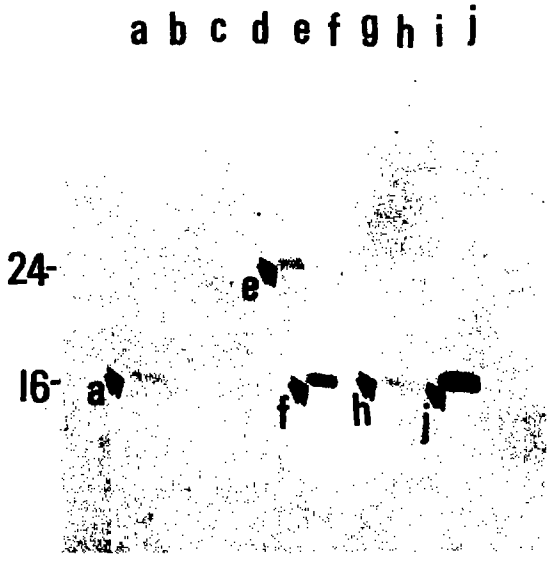

FIG. 2. SDS-PAGE AND IMMUNODETECTION OF MYOTOXIN AND CROSS-REACTING PROTEINS FROM DIFFERENT CROTALINE VENOMS.

Venoms separated in SDS-PAGE and electrophoretically transferred to nitrocellulose paper were immunoenzymatically detected with a conjugate of anti-myotoxin rabbit IgG – peroxidase and 3,3-diaminobenzidine as precipitable substrate. Fifty micrograms of *Agkistrodon bilineatus* venom (a); 50 μg of *Crotalus durissus durissus* venom (b); 50 μg of *Lachesis muta stenophrys* venom (c); 50 μg of *B. nasutus* venom (d); 50 μg of *B. picadoi* venom (e); 50 μg of *B. nummifer* venom (f); 50 μg of *B. ophryomegas* venom (g); 50 μg of *B. godmani* venom (h); 50 μg of *B. lateralis* venom (i); and 50 μg of adult *B. asper* venom from the Atlantic region (j). All samples were reduced with 2-mercaptoethanol. The numbers to the left of the SDS-PAGE indicate the molecular weight × 10^3.

Anti-myotoxin antibodies reacted with components present in the venoms of other crotaline species. *B. schlegelii* (Fig. 1), *B. godmani, B. nummifer* and *Agkistrodon bilineatus* venoms (Fig. 2) showed one band corresponding to 16,000 mol. wt, whereas *B. picadoi* venom (Fig. 2) presented one band of 24,000 mol. wt. Venom of *B. schlegelii* showed also a band of 15,000 mol. wt (Fig. 1). All these venoms gave a positive reaction when tested in native conditions by dot blotting.

Two-dimensional SDS-PAGE

Analysis of *B. asper* venom by overloaded two-dimensional SDS-PAGE combined with Western blotting showed the presence of myotoxin at the position of 16,000 mol. wt. No myotoxin aggregates of higher molecular weight were observed. Total protein stain of gels showed the migration of all components according to the corresponding molecular weight of the first dimension, without generating lower molecular weight bands in the second dimension (data not shown).

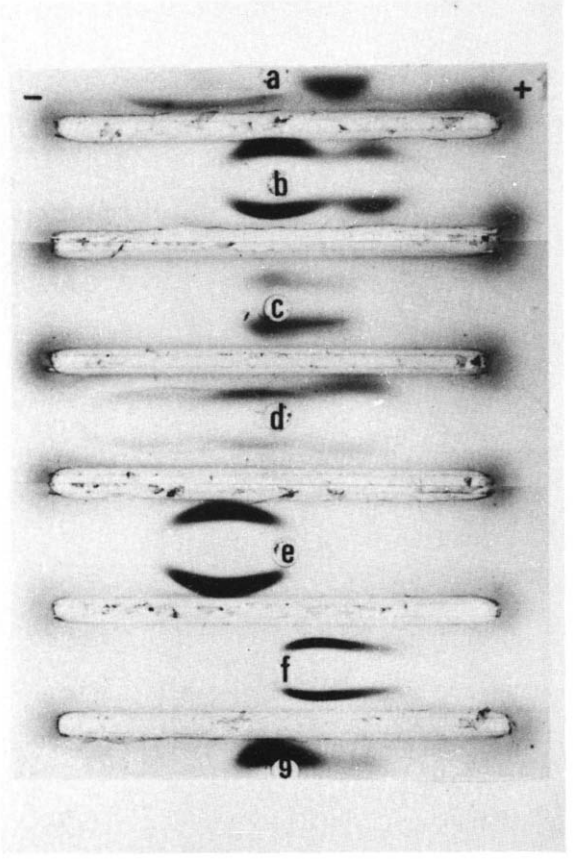

FIG. 3. IMMUNOELECTROPHORETIC ANALYSIS OF DIFFERENT CROTALINE VENOMS.
Rabbit monospecific serum anti-*B. asper* myotoxin (in the channels) was run against 75 μg/15 μl of electrophoretically-separated venoms of *A. bilineatus* (a), *B. godmani* (b), newborn *B. asper* from the Pacific region (c), *B. schlegelii* (d), *B. nummifer* (e), *B. picadoi* (f) and newborn *B. asper* from the Atlantic region (g).

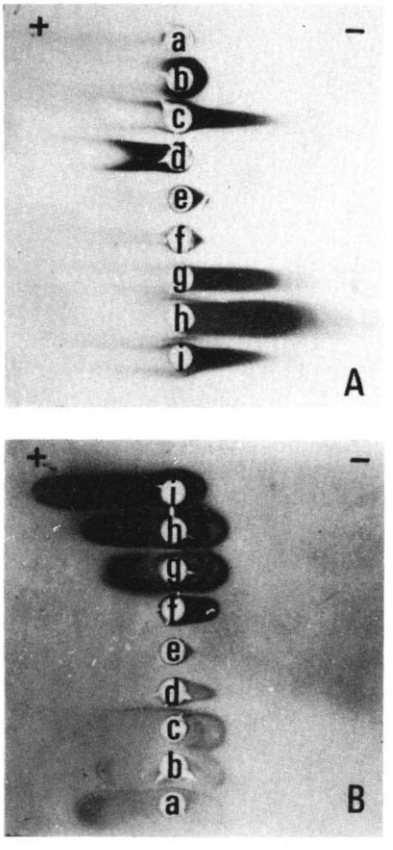

FIG. 4. ROCKET IMMUNOELECTROPHORESIS OF DIFFERENT CROTALINE VENOMS.
Monospecific sheep serum anti-*B. asper* myotoxin (A) and monospecific rabbit serum anti-*B. asper* myotoxin (B) were run against 50 μg/10 μl of venoms from *A. bilineatus* (a), *B. godmani* (b), *B. nummifer* (c), *B picadoi* (d), newborn *B. asper* from the Atlantic region (e); newborn *B. asper* from the Pacific region (f), adult *B. asper* from the Pacific region (g), adult *B. asper* from the Atlantic region (h), and *B. schlegelii* (i).

Immunoelectrophoretic analyses

Venoms which reacted with anti-myotoxin antibodies in Western blotting and dot blotting tests also reacted in immunoelectrophoresis (Fig. 3). *A. bilineatus* venom formed two diffuse and apparently independent precipitin arcs, one cathodic and one anodic, whereas *B. godmani* venom formed two precipitin arcs with total identity (Fig. 3). *B. nummifer* venom formed a single arc towards the cathode, whereas *B. picadoi* venom precipitated anodically as one arc (Fig. 3). A precipitin arc was observed with *B. asper* venoms from newborn specimens. Atlantic-newborn venom reacted near the origin, whereas Pacific-newborn venom showed a less intense reaction with a slightly anodic component (Fig. 3). *B. schlegelii* formed three diffuse precipitin arcs, two of them with total identity, whereas the cathodic arc showed a reaction of partial identity (Fig. 3).

By rocket immunoelectrophoresis, variations in the polarity of the precipitin reaction among the different venoms were also evident (Fig. 4). Some of the venoms precipitated with opposite polarity when tested against rabbit and sheep antisera (Fig. 4). By crossed

immunoelectrophoresis against sheep antiserum to myotoxin, *B. asper* venom showed two precipitation arcs with identical electrophoretic migration but different heights and intensities (data not shown).

DISCUSSION

B. asper myotoxin is a non-glycosylated, highly basic (pI 9.8 – 10.0), monomeric phospholipase A with a molecular weight of 16,000 by SDS-PAGE. Antibodies to *B. asper* myotoxin reacted with venoms from five crotaline species: *B. nummifer, B. schlegelii, B. godmani, B. picadoi* and *Agkistrodon bilineatus*. Thus, these venoms possess components which are antigenically related to *B. asper* myotoxin. Findings on the molecular weight and charge of these cross-reacting components indicated that they share a molecular weight of 16,000, with the exception of that of *B. picadoi* which has a molecular weight of 24,000. However, they exhibit a remarkable heterogeneity of charge, ranging from basic to acidic proteins. Venoms of *B. godmani, B. schlegelii* and *A. bilineatus* showed the presence of two to three cross-reacting proteins with different electrophoretic mobilities. The fact that in immunoelectrophoresis these venoms showed more cross-reacting components than in Western blotting might indicate an identical molecular weight of the various proteins. Alternatively, the loss of reactivity of some components in Western blotting due to the denaturing conditions inherent to the technique may occur. However, in the case of *B. schlegelii* venom, two bands of different molecular weight were observed in immunotransference. This agrees with the finding of several precipitin bands in immunoelectrophoresis.

B. asper myotoxin is a phospholipase A (GUTIERREZ *et al.*, 1984, 1986*a*), therefore the cross-reacting proteins studied could also have myotoxic and/or phospholipase A activities. Presently, we know that a purified protein from *B. nummifer* venom is a myotoxin devoid of phospholipase activity (GUTIERREZ *et al.*, 1986*b*). In the case of *A. bilineatus* venom, a myotoxic phospholipase A recently isolated has been shown to be a basic protein of 15,000 mol. wt (MEBS and SAMEJIMA, 1986). Its similar molecular weight to *B. asper* myotoxin, and the fact that the antibodies used react with a basic protein in *A. bilineatus* venom, suggest that the cross-reacting component could be the same described by MEBS and SAMEJIMA (1986).

Myotoxin was detected in different types of adult *B. asper* venoms as a single band of 16,000 mol. wt in Western blotting. By immunoprecipitation techniques, two superimposed parallel cathodic arcs were observed with *B. asper* venoms from adult specimens. However, it was shown that even a highly homogeneous sample of myotoxin generated the same two precipitin arcs. This finding correlates with previous studies using horse antibodies to myotoxin (LOMONTE *et al.*, 1985). It might be that the two precipitin arcs are due to two antigen – antibody systems with different diffusion rates, caused either by different classes of immunoglobulins or to the presence of aggregated myotoxin. Differences in *B. asper* venoms from newborn and adult specimens are not only quantitative, but also qualitative. The molecular weight of 'newborn-type' myotoxin(s) is close to that of 'adult-type', but the charge of the protein is considerably different, as evidenced by immunoelectrophoresis. Furthermore, venoms from newborn specimens presented an additional band of 18,000 mol. wt in Western blotting, not present in venoms from adults. A more detailed analysis of the 'newborn-type' myotoxin(s) is necessary in order to establish if this protein(s) is a 'precursor' of myotoxin, a different isotype or a cross-reacting component.

Rocket immunoelectrophoresis suggests that myotoxin is an abundant component in crude *B. asper* venom. This is supported by the observation of electrophoretic and immunoelectrophoretic patterns of crude venom. This agrees with previous studies which demonstrated that this is the most important myotoxic component in *B. asper* venom (LOMONTE *et al.*, 1985).

The difference in charge of the monospecific sheep and rabbit antibodies against *B. asper* myotoxin was an interesting finding since it demonstrates that the two animal species could elicit strikingly different antibody responses, although immunoglobulins from both species neutralize the toxin similarly. Furthermore, the myotoxin being a highly basic molecule, these results indicate that the antibodies produced are not necessarily of opposite charge.

In conclusion, our results indicate that myotoxin is a quantitatively important protein in crude *B. asper* venom, and that antigenically related components to this toxin exist in several other crotaline venoms from the genera *Bothrops* and *Agkistrodon*. *B. asper* myotoxin and its antigenically-related components seem to be part of a 'family' of proteins with similar molecular weights but different charges.

Acknowledgements — We thank LIZET M. TAYLOR for her technical help. E. MORENO and J. M. GUTIÉRREZ were research fellows from CONICIT of Costa Rica during this investigation. This project was supported by Grant 741-84-085 from the Vicerrectoría de Investigación, University of Costa Rica. Part of this work was presented by B. LOMONTE as partial fulfillment for the M.Sc. degree, University of Costa Rica.

REFERENCES

BERS, G. and GARFIN, D. (1985) Protein and nucleic acid blotting and immunobiochemical detection. *Biotechniques* 3, 276.

BOLAÑOS, R. and CERDAS, L. (1980). Producción y control de sueros antiofídicos en Costa Rica. *Boln of. Sanit. Panam.* 88, 184.

GUTIERREZ, J. M. and CHAVES, F. (1980) Efectos proteolítico, hemorrágico y mionecrótico de los venenos de serpientes costarricenses de los géneros *Bothrops, Crotalus* y *Lachesis*. *Toxicon* 18, 315.

GUTIERREZ, J. M. and CERDAS, L. (1984) Mecanismo de acción de miotoxinas aisladas de venenos de serpientes. *Rev. Biol. Trop.* 32, 213.

GUTIERREZ, J. M., OWNBY, C. L. and ODELL, G. V. (1984) Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. *Toxicon* 22, 115.

GUTIERREZ, J. M., LOMONTE, B., CHAVES, F., MORENO, E. and CERDAS, L. (1986*a*) Pharmacological activities of a toxic phospholipase A isolated from the venom of the snake *Bothrops asper*. *Comp. Biochem. Physiol.* 84c, 159.

GUTIERREZ, J. M., LOMONTE, B. and CERDAS, L. (1986*b*) Isolation and partial characterization of a myotoxin from the venom of the snake *Bothrops nummifer*. *Toxicon* 24, 885.

HERBERT, W. J. (1978) Mineral oil adjuvants and the immunization of laboratory animals. In: *Handbook of Experimental Immunology*, Vol. 3 (WEIR, D. M., Ed.). Oxford: Blackwell Scientific.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680.

LANGONE, J. J. (1982) Applications of immobilized protein A in immunochemical techniques. *J. immun. Meth.* 55, 227.

LOMONTE, B., GUTIERREZ, J. M. and MATA, E. (1985) Isolation from polyvalent antivenom of antibodies to a myotoxin in *Bothrops asper* snake venom. *Toxicon* 23, 807.

MEBS, D. and SAMEJIMA, Y. (1986) Isolation and characterization of myotoxin phospholipase A from crotalid venoms. *Toxicon* 24, 161.

NAKANE, P. K. and PIERCE, G. B. (1966) Enzyme-labelled antibodies: preparation and application for the localization of antigens. *J. Histochem. Cytochem.* 14, 929.

OUCHTERLONY, O. and NILSSON, L. A. (1978) Immunodiffusions and immunoelectrophoresis. In: *Handbook of Experimental Immunology*, Vol. I: *Immunochemistry*, p. 19.1 (Weir, D. M., Ed.). Oxford: Blackwell Scientific.

OWNBY, C. L. (1982) Pathology of rattlesnake envenomation. In: *Rattlesnake Venoms, Their Actions and Treatment*, p. 163 (TU, A. T., Ed.). New York: Marcel Dekker.

RAMIREZ, P., BONILLA, J. A., MORENO, E. and LEON, P. (1983) Electrophoretic transfer of viral proteins to nitrocellulose sheets and detection with peroxidase-bound lectins and protein A. *J. immun. Meth.* 62, 15.

REISFELD, R. A., LEWIS, U. J. and WILLIAMS, D. E. (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* **195**, 281.

TOWBIN, H. and GORDON, J. (1984) Immunoblotting and dot immunobinding: current status and outlook. *J. immun. Meth.* **72**, 313.

TOWBIN, H., SAEHELIN, J. and GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5340.

WEEKE, B. (1973) Rocket immunoelectrophoresis. In: *Manual of Quantitative Immunoelectrophoresis*, pp. 37, 47 (AXELSEN, N. H., KROLL, J. and WEEKE, B., Eds). Oslo: Universtetsforlaget.