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A NEW METHOD FOR THE DETECTION OF PHOSPHOLIPASE A_2 VARIANTS: IDENTIFICATION OF ISOZYMES IN THE VENOMS OF NEWBORN AND ADULT *BOTHROPS ASPER* (TERCIOPELO) SNAKES

EDGARDO MORENO,[1,2]* ALBERTO ALAPE,[1] MANUEL SÁNCHEZ[1] and JOSÉ MARIA GUTIÉRREZ[3]

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

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E. MORENO, A. ALAPE, M. SÁNCHEZ and J. M. GUTIÉRREZ. A new method for the detection of phospholipase A_2 variants: identification of isozymes in the venoms of newborn and adult *Bothrops asper* (terciopelo) snakes. *Toxicon* **26**, 363–371, 1988. — A new method for the identification of phospholipase A_2 isozymes in snake venoms is described. The technique is based on the separation of the venom components by isoelectric focusing in agarose gels, transfer of the protein bands by diffusion onto nitrocellulose paper and detection of the phospholipolytic activity of the enzymes by a hemolytic assay either in agarose gels or by benzidine reaction on a solid matrix. Striking differences in the electrophoretic patterns of the phospholipase A_2 isozymes between the Atlantic and Pacific venoms and between the newborn and adult venoms from *Bothrops asper* specimens were observed. The method allowed the detection of 9 different phospholipase A_2 isozymes in the venom of adult Atlantic, 7 isozymes in the venom of adult Pacific, and 2–3 isozymes in the venoms of newborn specimens. Horse polyvalent antivenom varied in its capacity to neutralize the phospholipolytic activity of the different isozymes in the same venom and among different venoms.

INTRODUCTION

SNAKE VENOMS constitute a rich source of phospholipases A_2 (TU, 1977). Whereas many of them are not toxic, there is a growing list of phospholipases A_2 which exert toxic effects such as neurotoxicity, myotoxicity and anticoagulability (ROSENBERG, 1979). A single venom usually has several phospholipase A_2 isozymes which differ in their charge and/or molecular weight. Although chromatographic procedures allow for the isolation of phospholipase A_2 isozymes (TU, 1977), electrophoretic techniques permit a rapid screening of these proteins in crude venoms and other biological fluids (SALACH *et al.*, 1971; SHILOAH *et al.*, 1973; SHIER and TROTTE, 1978; DURKIN *et al.*, 1981; DUBOURDIEU *et al.*, 1987).

We have developed a simple and sensitive assay based on the separation of venom components by means of isoelectric focusing (IEF), transference onto nitrocellulose paper, and identification of phospholipase A_2 isozymes by an indirect hemolytic assay in agarose gels. The new method is described in this communication, together with a

*To whom correspondence should be addressed.

comparative study of the phospholipase A_2 isozymes in venoms from newborn and adult specimens of *Bothrops asper*. We also describe the use of this procedure in the study of the neutralization of phospholipases A_2 by antivenoms.

MATERIALS AND METHODS

Venoms and antivenoms

Venoms were obtained from adult and newborn *Bothrops asper* snakes. The adult specimens were captured either in the Atlantic or in the Pacific regions of Costa Rica. Newborn specimens were born at the serpentarium of the Instituto Clodomiro Picado. Venoms from adult and newborn snakes from each region were pooled separately, lyophilized and kept at −20°C until used. Polyvalent and anticoral antivenoms were produced in horses, purified and characterized as described elsewhere (BOLAÑOS and CERDAS, 1980).

Isozyme separation

Phospholipase A_2 isozymes were separated in isoelectric focusing electrophoresis (IEF) following instructions in the Pharmacia manual, using a flat bed IEF apparatus from Pharmacia (FBE 3000) with a cooling system (Coolnics CTR-220, Japan) at 4°C in combination with an electrophoretic power supply ECPC 3000/150 (Pharmacia), for 1500 Vh. For IEF, 20 ml of 1% agarose IEF (Pharmacia) in double distilled water containing 12% sorbitol (Sigma) and 5% Pharmalyte (Pharmacia) range pH 3 − 10, was poured onto a glass plate (11.5 cm × 4 cm) previously coated with a dry thin layer of 0.5% agarose IEF. A continuous slot in the gel was made by placing a teflon sheet (1 mm × 6 cm) in the middle of the glass plate before the gelling of the agarose. Before use, the gel was kept in a moist chamber for 1 hr at 4°C. One mg of venom, dissolved in 150 µl of double distilled water containing 5% Pharmalytes was delivered in the continuous well. After IEF, a strip of agarose gel across the electrodes was cut out and the pH gradient determined in 0.5 cm gel sections soaked in 1 ml of double distilled water.

Blotting and detection

The transfer to nitrocellulose paper was carried out essentially as described by SITTENFELD and MORENO (1987). A sheet of nitrocellulose paper soaked in transference buffer (0.025 M Tris, 0.193 M glycine, 20% methanol, pH 8.3; TOWBIN *et al.*, 1979) was placed over the gel, previously covered with 10 ml of the same buffer. Air bubbles between the gel and the paper were carefully removed. The blotting was performed by covering the nitrocellulose with 4 sheets of filter paper, 10 sheets of paper towel, and a glass plate, under 2 kg pressure for 30 min at room temperature. After transference, the nitrocellulose paper was rinsed and incubated in coating buffer (0.07 M barbiturate buffer, pH 8.6, 1% casein hydrolysate) for 2 hr at room temperature.

For the detection of the phospholipase A_2 isozymes, the nitrocellulose sheet was sliced into 0.5 cm strips which were placed 1 cm apart over a plate (11 cm × 5 cm) with a substrate gel (0.5% agarose type II from Sigma, dissolved in phosphate-buffered saline, pH 7.4, containing 4% of packed human erythrocytes, 4% of egg yolk as source of lecithin and 1% of $CaCl_2$). The plate was kept at 37°C in a humid chamber and the nitrocellulose strips removed from the gel at 1 hr or 3 hr of incubation. After removal of the strips, the substrate gel was reincubated at 4°C for additional 3, 7, 12, 15 and 21 hr. The plate was photographed at the different time intervals with a yellow filter, in order to increase contrast. Alternatively, the peroxidase activity of the hemoglobin bands impregnating the nitrocellulose strips was developed with 0.5 mg/ml of benzidine (Sigma) dissolved in 0.02 M Tris − HCl, pH 7.2 containing 0.01% of H_2O_2.

Neutralization by antivenom

After transference, the free sites remaining on the nitrocellulose paper were blocked for 1 hr at room temperature with coating buffer. The strips were incubated for 3 hr at room temperature with different dilutions in phosphate-buffered saline (pH 7.4) of the polyvalent antivenom or undiluted anticoral antivenom as control antiserum. After neutralization each strip was tested for phospholipase A_2 activity as described above.

RESULTS

Optimal setting for IEF of complete *B. asper* venoms separated under native conditions was found to be at 15 W, 650 V and 30 mA using 0.05 M H_2SO_4 and 1 M NaOH as anode and cathode electrode solutions, respectively. Blotting of IEF separated venom proteins was performed immediately after electrophoresis in order to avoid diffusion of the bands in the agarose gel. Amounts of venom lower than 1 mg per gel (11.5 cm × 7 cm) were completely transferrred to the nitrocellulose sheet. Higher amounts of venom did not

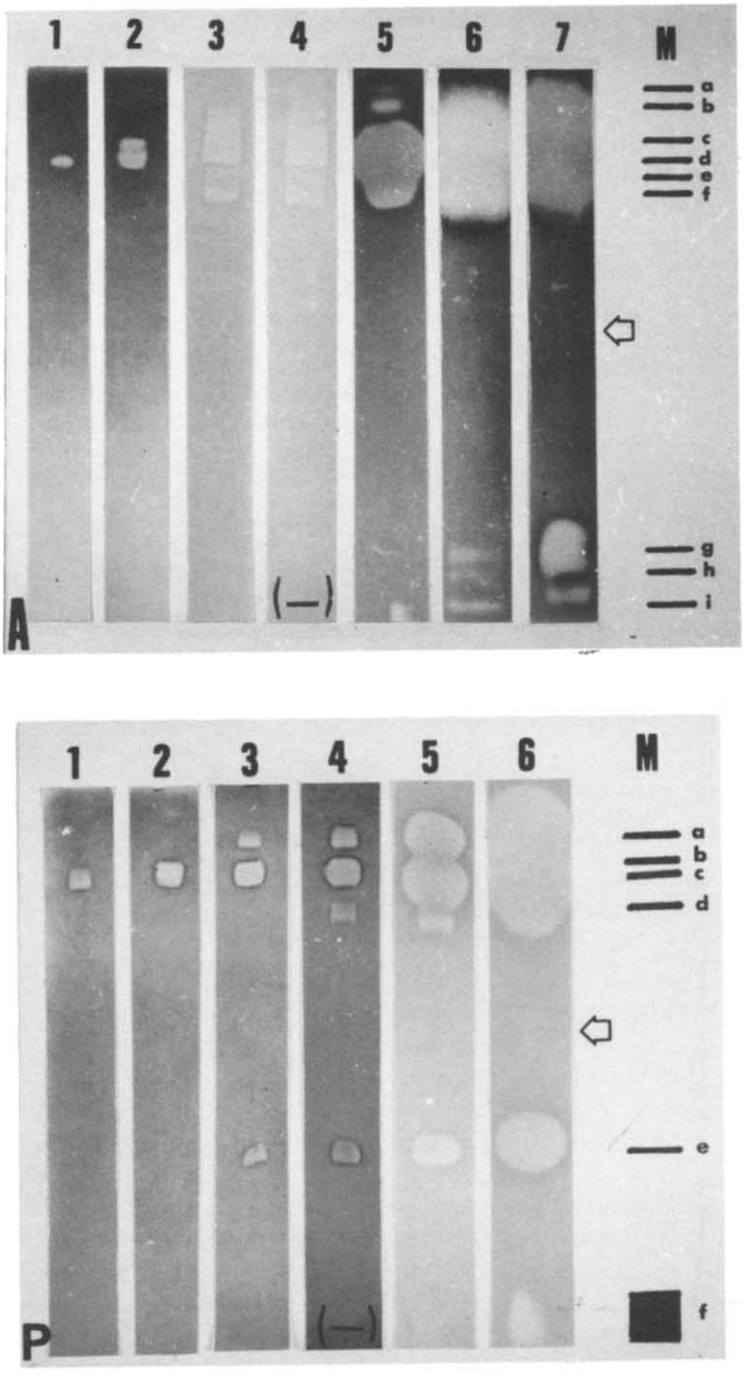

FIG. 1. PHOSPHOLIPASE A_2 ELECTROPHORETIC VARIANTS FROM ADULT *Bothrops asper* VENOMS. Atlantic venom (A) or Pacific venom (P) electrophoretically separated and transferred onto nitrocellulose paper were tested for phospholipolytic activity on substrate gels at 37°C for 1 (1) and 3 hr (2), and then at 4°C for an additional 3 (3), 7 (4), 12 (5), 15 (6) and 21 hr (7). Diagrams (M) specify the number and relative mobility of the different isozymes. The arrow shows the application point.

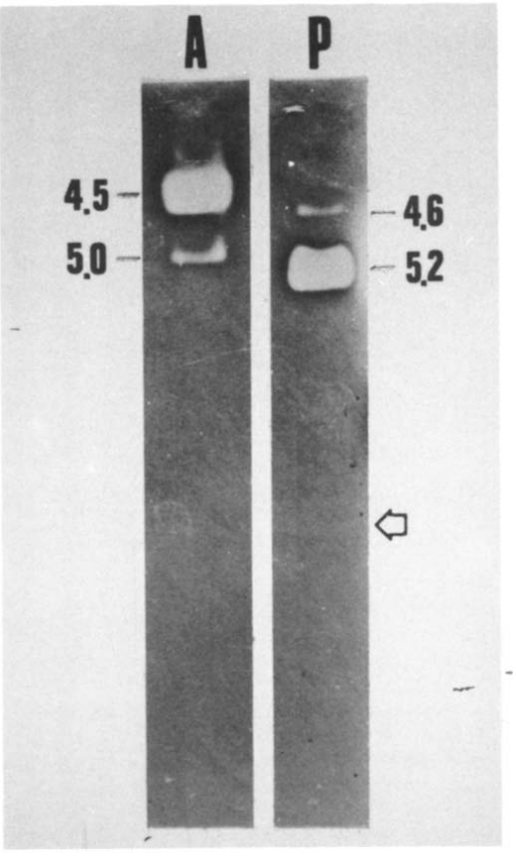

FIG. 2. PHOSPHOLIPASE A_2 ELECTROPHORETIC VARIANTS FROM NEWBORN *Bothrops asper* VENOMS. Atlantic venom (A) or Pacific venom (P) electrophoretically separated and transferred onto nitrocellulose paper were tested for phospholipolytic activity on substrate gels at 37°C for 3 hr and at 4°C for 1 hr. Numbers are the isoelectric pH at which the bands migrate. The arrow shows the application point.

show additional bands with phospholipase A_2 activity and saturated the protein binding capacity of the nitrocellulose paper for the most abundant isozymes (bands c – f in the adult Atlantic venom, bands a – e in the adult Pacific venom, band at pH 4.5 in the newborn Atlantic venom and band at pH 5.2 in the newborn Pacific venom). The resolution of the bands improved when concentrations between 0.25 mg and 0.1 mg of venom per gel were transferred, however some of the bands with weak phospholipase A_2 activity (or low concentration of phospholipase A_2) did not appear in the hemolytic test. Blotting buffer for Western blot transfers (TOWBIN *et al.*, 1979) was more efficient than phosphate-buffered saline, pH 7.4. The same blotting efficiency was observed when nitrocellulose of pore sizes of 0.2 μm or 0.45 μm were used. No differences in the results were observed with blotting temperatures ranging from 4 to 37°C. Decreased resolution of the blotting system was obtained when the transference time exceeded 45 min. Blotting times shorter than 30 min did not allow complete transference. The nitrocellulose strips could be kept dry at 4°C for up to one week without diminishing the phospholipase A_2 activity. Faster appearance of the bands occurred at erythrocyte concentrations from 2 to 3%, however, the resolution of the bands improved when erythrocyte concentrations from 4 to 6% were used. After removal of the nitrocellulose strips from the substrate gel,

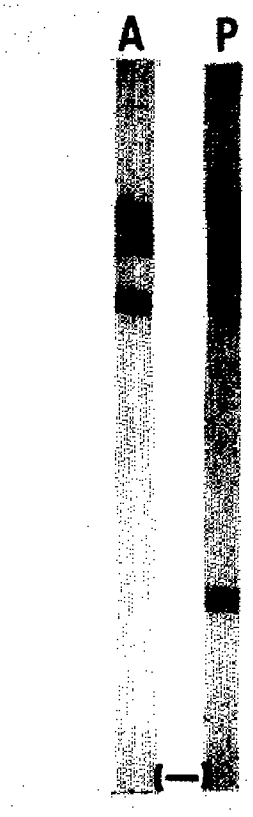

FIG. 3. PHOSPHOLIPASE A_2 ELECTROPHORETIC VARIANTS FROM ADULT *Bothrops asper* VENOMS ON NITROCELLULOSE PAPER.
Atlantic venom (A) or Pacific venom (P) electrophoretically separated and transferred onto nitrocellulose paper were tested for phospholipolytic activity on substrate gels at 37°C for 3 hr. The nitrocellulose strips were coated and the peroxidase activity of hemoglobin on the paper detected with benzidine in the presence of H_2O_2.

the hemolytic reaction continued concomitantly with the appearance of new bands and ending with fusion of the individual bands (Fig. 1). In this system, hemolysis is the consequence of phospholipase A_2 activity and not direct hemolytic activity, since venoms incubated on substrate gels without egg yolk did not induce hemolysis.

The hemolytic patterns at various time intervals of phospholipases A_2 from venoms of adult *B. asper* are presented in Fig. 1. Striking qualitative and quantitative differences between Atlantic and Pacific venoms are observed. Six bands with anodic mobility (ranging from pH 4 to 5.4) and 3 bands with cathodic migration (ranging from pH 9.3 to pH 10) are resolved in the Atlantic venom. In contrast, 4 bands with anodic mobility (ranging from pH 4.3 to pH 5.4), one band with neutral migration (pH 7.2) and one diffuse band with cathodic migration (ranging from pH 9.5 to pH 10) are present in the Pacific venom of adult *B. asper*. The different incubation times as well as the alternative times at which the nitrocellulose strips are removed from the substrate gel allow the detection and resolution of different phospholipase A_2 bands.

TABLE 1. NEUTRALIZATION OF THE PHOSPHOLIPOLYTIC ACTIVITY BY POLYVALENT ANTIVENOM OF THE DIFFERENT PHOSPHOLIPASE A_2 ELECTROPHORETIC VARIANTS FROM VENOMS OF *Bothrops asper**

Snake venom	Isozyme band	Polyvalent antivenom dilution				
		1/1	1/5	1/10	1/25	1/100
Adult Atlantic	a	−	+	+ +	+ + +	+ + + +
	b	−	+	+ +	+ + +	+ + + +
	c	+	+ +	+ + +	+ + + +	+ + + + +
	d	+	+ +	+ + +	+ + + +	+ + + + +
	e	+	+	+ + +	+ + +	+ + + +
	f	+	+	+ + +	+ + +	+ + + +
	g	−	−	−	−	+
	h	−	−	−	−	+
	i	−	−	−	−	+
Adult Pacific	a	−	−	−	+	+ +
	b	−	+	+ +	+ + +	+ + + +
	c	−	+	+ +	+ + +	+ + + +
	d	−	−	−	−	+
	e	−	−	+	+ +	+ + +
	f	−	−	−	−	+
Newborn Atlantic	pH 4.5	+ +	+ + +	+ + + +	+ + + + +	+ + + + +
	pH 5.0	−	−	+	+ +	+ + +
Newborn Pacific	pH 4.6	−	−	+	+ +	+ + +
	pH 5.2	+ +	+ + +	+ + + +	+ + + + +	+ + + + +

*The evaluation of the test was performed by grading the intensity of each hemolytic band from (−) to (+ + + + +) comparatively with the same band corresponding to the control strip which was always graded as (+ + + + +). Each hemolytic band in strips incubated with antivenom was analyzed at the time in which the hemolytic band of the control was clearly resolved, e.g. in the control test of the adult Atlantic venom, bands "a" and "b" were clearly resolved after 18 hr, while in the neutralization test, in which polyvalent antivenom was not diluted, the same bands did not appear at this time. The control strip was incubated with undiluted anticoral antivenom which does not neutralize phospholipase A_2 activity of *B. asper* venoms. During the first 3 hr the test was carried out at 37°C, afterwards the plates were incubated at 4°C.

The hemolytic patterns of newborn Atlantic and Pacific *B. asper* venoms are shown in Fig. 2. Two bands with anodic mobility are present, although the proportions between them vary: in the Atlantic venom the most anodic band is more abundant than the more cathodic one; on the contrary, in the Pacific venom the most anodic band is less abundant than the more cathodic one. In some electrophoretograms, the thick band in both venoms seemed to be the result of the fusion of two bands. The bands on the substrate gel were resolved after 3 hr of incubation at 37°C and 1 hr of incubation at 4°C. No additional electrophoretic variants were observed when these venoms were incubated for an additional 21 hr at 4°C.

The nitrocellulose transfers of adult venoms revealed with benzidine are shown in Fig. 3. The strips correspond to 3 hr of incubation at 37°C. At this time the cathodic bands are not visible; however, if the strips are incubated with the substrate gel for 15 hr at 4°C, the cathodic phospholipases A_2 appear at the bottom of the paper (not shown). The sensitivity of the system is slightly higher than that obtained with the substrate gel, e.g. band "d" from Fig. 1P is present in the strip only after 6 − 10 hr of incubation, while in the nitrocellulose paper it could be detected after 3 hr of incubation (Fig. 3P). Since the nitrocellulose paper placed on the substrate gel induces slight hemolysis (only on those erythrocytes in direct contact with the paper), incubation times longer than 3 hr slowly increased the background of the paper and reduced the sharpness of the bands on the nitrocellulose due to the diffusion of hemoglobin through the gel. As previously demonstrated (RAMIREZ *et al.*, 1983), the intensity of the bands can be quantitated by

making the nitrocellulose strips transparent after immersion in xylol and measuring the color in a gel densitometer (not shown).

The neutralization of the phospholipase A_2 isozymes from adult and newborn *B. asper* venoms with polyvalent antivenom is presented in Table 1. Clearly, some of the bands were better neutralized than others. In the adult venoms the cathodic bands were better neutralized than the neutral (in the Pacific venom) or the acidic bands. The neutralization of the major isozymes from newborn venoms was more difficult to achieve than that of the adult isozymes. In grading the intensity of hemolysis, each hemolytic band was analyzed at the time when that particular band in the control was clearly resolved. In some cases, however, if substrate gels were incubated for an additional time at 4°C, some of the bands slowly reappeared in the gel.

DISCUSSION

The method described in this communication is highly useful in identifying the various phospholipase A_2 isozymes present in biological fluids in general and snake venoms in particular. Moreover, since it gives information on the isoelectric pH of the protein variants, it could help in choosing the chromatographic methods for purification of phospholipases A_2. Since the separation is carried out under native conditions, the procedure allows not only a qualitative identification, but also a semiquantitative estimation of the activity of the fractions. The method has several advantages over other techniques: it is simple and relatively inexpensive, it has high resolution based on the IEF separation, and the protein bands on the nitrocellulose strips can be detected by immunoenzymatic or immunoradiometric procedures (SITTENFELD and MORENO, 1987). Furthermore, the bands on the nitrocellulose sheet can be quantitated by densitometry (RAMIREZ *et al.*, 1983). When the strips are placed on the agarose – erythrocyte gel, part of the hemoglobin released after cell lysis binds to the nitrocellulose, and this can be detected on the basis of the peroxidase activity of hemoglobin. In this way, strips can be stored for long periods of time for estimation by densitometry. Moreover, the method can be easily adapted for the detection of other enzymes if an adequate precipitable substrate is added to the nitrocellulose transfer.

The technique described here is valuable in phylogenetical as well as ontogenetical studies of snake venoms. For instance, when electrophoretograms of Atlantic and Pacific venoms from newborn and adult specimens of *B. asper* were compared, interesting qualitative and quantitative variations were observed. The results agree with a previous study (GUTIÉRREZ *et al.*, 1980), which showed that venoms from adult *B. asper* have a significantly higher indirect hemolytic activity than those from newborn snakes. Our findings in the present work demonstrate that the venoms from newborn specimens contain 2 – 3 acidic phospholipase A_2 isozymes, however, striking differences in the migration of the major and minor isozymes were evident. Moreover, there are interesting variations between the Atlantic and Pacific populations of both newborn and adult specimens. Particularly in the adult Pacific venom there is one isozyme which focuses at pH 7.2 and which is absent in the Atlantic samples. In previous work, conspicuous electrophoretic, chromatographic and pharmacologic differences between *B. asper* venoms from Atlantic and Pacific populations were demonstrated (JIMENEZ-PORRAS, 1964; GUTIÉRREZ *et al.*, 1980; ARAGÓN and GUBENSEK, 1981).

Previous biochemical studies have shown the presence of 3 acidic and 2 basic phospholipases A_2 in the venom of *B. asper* (FERLAN and GUBENSEK, 1978; ALAGÓN *et*

al, 1980; GUTIÉRREZ *et al*, 1984; MEBS and SAMEJIMA, 1986). Our findings demonstrate the presence of several acidic phospholipases A_2 (6 in the Atlantic venom and 4 in the Pacific venom) and at least 3 basic phospholipases in the Atlantic venom (in the Pacific venom the basic variants were not easily resolved). These data are in agreement with recent findings by LOMONTE *et al.* (in preparation), who identified 2 phospholipases A_2 with cathodic migration in *B. asper*. Although the basic phospholipases A_2 do not exert a high enzymatic activity, they are relatively abundant in the crude venom of adult specimens (LOMONTE *et al.*, 1987). It might be that one of the basic variants corresponds to the myotoxin that has been studied previously (GUTIÉRREZ *et al.* 1984, 1986). The acidic components of this venom do not exert a relevant myotoxic activity (GUTIÉRREZ *et al.*, 1984) despite their high phospholipase A_2 activity, an indication that phospholipolytic action *per se* does not necessarily imply myotoxicity.

The method described may also be used to study the neutralization of phospholipase A_2 activity by antivenoms. In this regard, it has been stated that the problem of using crude venoms to study their neutralization by antivenoms is that many toxic and enzymatic activities are due to several isoproteins present in a particular venom, and that it is not possible to discern discrete toxin–antitoxin systems when working with crude venoms (OHSAKA, 1979). Although this is not a quantitative technique, it allows the study of neutralization of individual isozymes in semiquantitative terms; this can be done by determining the highest dilution of antivenom that neutralized each isozyme. Results showed that some isozymes are better neutralized than others (e.g. the basic phospholipases A_2, bands "e" and "f" in the Atlantic and "d" in the Pacific adult venoms and the minor band in the newborn venoms). Thus, this technique can be used to study the specificity of antiserum to each of the isozymes in a particular snake venom. Interestingly, the observation of the hemolytic bands in the substrate gel did not only depend on the concentration of the neutralizing serum, but also depended on the time at which the test was monitored. Therefore, some of the bands which were neutralized after 10 hr of incubation, were not neutralized after 15 hr. This result contrasts with the semiquantitative neutralization studies of the phospholipase A_2 activity carried out with complete venoms by the radial hemolytic test in substrate gels and the phospholipolytic tube assay using lecithin as substrate (GENÉ *et al.*, 1985; GUTIÉRREZ *et al.*, 1987), where the neutralization of the activity is time independent. This difference might be due to the fact that the proteins bound to the nitrocellulose matrix may alter their conformation (SITTENFELD and MORENO, 1987) avoiding complete neutralization. On the other hand, liquid phase neutralization assays carried out in tubes usually produce large aggregates which may exclude the possibility of contact between the active enzymatic components and the substrate system.

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