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RABBIT IgG ANTIBODIES AGAINST PHOSPHOLIPASE A_2 FROM *CROTALUS DURISSUS TERRIFICUS* NEUTRALIZE THE LETHAL ACTIVITY OF THE VENOM

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Abstract *Crotalus durissus terrificus* (*C.d.t.*) (South American rattlesnake) venom possesses myotoxic and neurotoxic activities, both of which are also expressed by crotoxin, the principal toxin of this venom. Crotoxin contains a basic phospholipase A_2 (PLA_2) and a non toxic acidic protein, crotapotin. We have produced and investigated the ability of IgG antibodies raised in rabbits against PLA_2 to neutralize the lethality of the whole venom. PLA_2 was isolated by gel filtration chromatography (Sephadex G-75). Specific antibodies were obtained by subcutaneous and intramuscular inoculation of PLA_2 (700 µg) with Freund adjuvant. Groups of six mice (20 $\pm$ 2 g) were inoculated with 0.5 ml i.p. of *C. d. t.* venom (4 µg) or a mixture of venom that had been preincubated with the desired volume of IgG antibodies. Mortality, recorded 24 and 48 h after inoculation, showed that IgG anti-PLA_2 were more effective than anticrotalic serum in neutralizing the lethal activity. These results demonstrate that it could be possible to obtain an anti-venom made by specific antibodies with a high level of protection against the lethal component of *C.d.t*. venom, and/or the inclusion of these antibodies as a supplement in heterologous anti-venoms.

Key words: *Crotalus durissus terrificus,* phospholipase A_2, antibodies IgG, neutralization

Resumen ***Los anticuerpos IgG de conejos anti-fosfolipasa A_2 de* Crotalus durissus terrificus *neutralizan la actividad letal del veneno***. El veneno de *Crotalus durissus terrificus* (*C.d.t.*) (Cascabel de Sud América) posee actividad miotóxica y neurotóxica, actividades que también exhibe el complejo crotoxina, principal componente tóxico de este veneno. El complejo crotoxina está constituido por una fosfolipasa A_2 básica (PLA_2) y una proteína acídica no tóxica, el crotapotín. En este trabajo se estudió la capacidad neutralizante de anticuerpos IgG anti-PLA_2 sobre la letalidad inducida por el veneno entero. El antígeno PLA_2 fue aislado por cromatografía de filtración en gel (*Sephadex* G-75). Se inocularon conejos machos por vía subcutánea e intramuscular, con 700 µg de PLA_2 y adyuvante para la obtención de anticuerpos específicos. La capacidad neutralizante del antisuero se analizó en ratones por inoculación con diluciones de veneno entero preincubado con un volumen adecuado de anticuerpos IgG anti-PLA_2. Se inocularion ratones controles con 0.5 ml i.p. de veneno (4 $\mu g.ml^{-1}$). El número de muertes fue contabilizado a las 24 y 48 h posteriores a la inoculación, demostrándose que la capacidad neutralizante de los anticuerpos IgG anti-PLA_2 fue superior a la obtenida con el antiveneno crotálico. Los resultados obtenidos demuestran la potencial aplicación de antivenenos constituidos por anticuerpos específicos contra PLA_2, y/o la inclusión de estos anticuerpos como suplementos en antivenenos polivalentes.

Palabras clave: *Crotalus durissus terrificus*, fosfolipasa A_2, anticuerpos IgG, neutralización

Crotoxin, the major toxin of South American rattlesnake (*Crotalus durissus terrificus*) (*C.d.t.*) venom[1], is a potent phospholipase A_2 neurotoxin that produces neuromuscular blockade[2]. Several additional biological activities, including myotoxicity[3], hemolysis[4] and platelet aggregating activity[5], have also been attributed to crotoxin.

Crotoxin consists of a basic, weakly toxic phospholipase A_2 (PLA_2) subunit and an acidic, non-toxic subunit (crotapotin) devoid of enzymatic activity[6,7]. Crotapotin acts as a chaperon in the complex by preventing non-specific binding of PLA_2, thereby potentiating its toxicity[8]. Thus, the stability of the interaction between PLA_2 and crotapotin plays a major role in the toxicity of crotoxin[9].

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Up to now, heterologous antibody administration has been the treatment of choice for snakebite victims. This practice requires the production, normally by horse immunization, of a high-titer anti-venom serum; semi purified globulin preparation or also fraction of antibodies, all of them against the whole venom[10-13]. Owing to the large amount of heterologous proteins administered, serum therapy may induce adverse reactions[12, 13]. In these circumstances specific antibodies against the lethal component of the venom would be more appropriate for immunotherapeutic applications.

In the present study, we examined the ability of specific IgG rabbit antibodies against PLA$_2$ (IgG anti-PLA$_2$) from *C.d.t.* venom to neutralize the lethal activity of this venom.

Materials and Methods

Venom and toxin (antigen)

Desiccated *C.d.t.* venom was obtained from *Zoológico de la Ciudad de Corrientes*. PLA$_2$ was purified from *C.d.t.* venom by gel filtration chromatography. The procedure was carried out in one step using Sephadex G-75 (30x1 cm) pre-equilibrated with 20 mM glycin, 150 mM NaCl, pH 1.9, according to a modification of the method described by Landucci et al.[5]. Fractions of 0.75 ml/tube were collected at a flow rate of 12 ml/h.

Animals

Male Swiss white mice weighing 20-22 g were supplied by the animal facility of the *Facultad de Ciencias Veterinarias*, UNNE. The mice were housed at 25 °C on a 12 h light/dark cycle and had free access to food and water.

Male New Zealand white rabbits weighing 3 kg were housed individually with free access to food and water.

Anti-venom

The anti-crotalic venom used was a semi-purified immunoglobulin-rich solution produced by hyperimmunization of horses with *C.d.t*. venom provided by ANLIS (*Instituto Nacional de Producción de Biológicos. Administracion Nacional de Laboratorios e Institutos de Salud "Dr. Carlos Malbrán*, Buenos Aires, Argentina), and designated ACS (anti-crotalic serum).

Anti-sera raised in rabbits and IgG anti-PLA$_2$

Rabbits were immunized by successive intramuscular and subcutaneous inoculations with 700 µg of PLA$_2$ per rabbit. The first injections included Freund's complete adjuvant (*Sigma*) in a 1:1 ratio. The subsequent boosters were given in the same way, but using Freund´s incomplete adjuvant with the toxin dissolved in PBS.

The antibody levels in the sera were monitored by gel immunodiffusion[14] and ELISA[15]. Blood samples were collected from a marginal ear vein and stored at 4 °C. The sera were subsequently separated by centrifugation and aliquots were stored at –70 °C.

IgG anti-PLA$_2$ were obtained by ammonium sulfate precipitation, desionizated by exclusion molecular chromatography, Sephadex G-25 column (20 x 0.75 cm) equilibrated with 10 mM phosphate buffer pH 7.2. The desionizated IgG were then stored at –70 °C.

Antibody detection

Immunodiffusion assay

Double immunodiffusion was carried out on Petri plates with a base of 2% w/v agar coated with a shell of 1g of agar in 100 µl of 145 mM H_3BO_3, 50 mM NaOH pH 8.6. Pairs of wells were filled with 50 µl of 1 mg.ml^{-1} antigen (PLA$_2$), and 50 µl of ACS, anti-sera raised in rabbits or IgG anti–PLA$_2$. Immunodiffusion was allowed to proceed for 48 h at 4 °C until immunoprecipitin bands were observed.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (96 wells) were coated overnight at 4 °C with 100 µl of PLA$_2$ (3 µg.ml^{-1}) in phosphate-buffered saline (PBS). The plates were washed three times with PBS containing 0.5% Tween 20 (PBS/Tween) and unbound sites were blocked for 1 h at room temperature with 2% bovine casein in (PBS).

The plates were washed three times with PBS/Tween and used immediately for ELISA. To measure the serum titers, 100 ml of serial dilutions of serum (ACS or rabbit anti-sera; 80 mg.ml^{-1} of initial protein concentration which is used in anti-venom therapy routine) were added to the plates and incubated for 1 h at 37 °C. The plates were then washed and incubated for 1 h with 100 µl of a goat anti-rabbit IgG–peroxidase conjugate (*Sigma*, 1:10 000 in PBS), followed by further washing. The substrate solution for the peroxidase assay (H_2O_2/OPD) was added and the enzymatic reaction allowed proceeding for 15 min at room temperature in the dark. The reaction was stopped with 50 µl of H_2SO_4 3N and the absorbance was read at 492 nm with a *SpectraMax* 340 multi-well plate reader.

Immunoblotting

Antigen (PLA$_2$) and whole venom (1 mg.ml^{-1}) were separated on 12.5% in SDS-PAGE at 200 V for 45 min and the proteins then transferred electrophoretically to nitrocellulose membranes (0.45 mm) in a transfer tank at 300 mA for 1 h. Subsequently, the membranes were blocked at room temperature for 2 h in a solution of 5% non-fat milk/0.05% Tween 20. After washing three times in Tris-buffered saline (TBS; 0.01 M Tris–HCl, 0.17 M NaCl, pH 7.6), the membranes were incubated overnight with rabbit anti-serum, IgG anti-PLA$_2$ or ACS (diluted 1:1 000; 0,1 mg.ml^{-1}; 1:2 000 in TBS, respectively). After washing again with TBS, bound antibodies were detected with a goat anti-rabbit IgG peroxidase conjugate (*Sigma*; 1:1 000 in TBS) for rabbit anti-serum or with a rabbit anti-horse IgG peroxidase conjugate (*Sigma*; 1:1 000 in TBS) for ACS for 1 h at room temperature with shaking. At the end of this incubation, the blots were washed, developed with 4-chloro-1-naphthol (*Sigma*; 0.03% in 0.05 M Tris-HCl, pH 7.6, containing 0.03% H_2O_2/OPD) and documented.

Hemolytic activity neutralization

In order to evaluate the ability of the IgG anti-PLA$_2$ to neutralize the phospholipase activity of the venom, an assay to determine the indirect hemolytic activity was carried out[16]. Twenty five milliliters of 1% (w/v) agar in PBS (pH 8.1) containing 0.3 ml of packed sheep erythrocytes, 0.3 ml of egg yolk in saline solution (1:3) and 0.25 ml of 0.01 M $CaCl_2$, was applied to plastic plates (135 x 80 mm) and allowed to gel. Then, 3 mm diameter wells were filled with 15 µl of venom or venom preincubated with different amount of ACS or IgG anti-

PLA_2. One hemolytic dose (HD) was defined as the lowest amount of venom (µg) which induces a hemolytic halo around the well of 19 mm. The PLA_2 neutralizing ability of the anti-sera was expressed as ED_{50} defined as the amount of anti-venom (mg of protein) needed to reduce by 50% the hemolytic activity of the whole venom.

Lethal activity neutralization

Neutralization of lethality was performed by incubating different mixtures of venom and antivenom at 37 °C for 30 min. Then 0.5 ml of the mixture was injected i.p. into mice (20 ± 2g, 6 mice per group) and animals were observed for 48 h. Each mouse received 4 µg of venom, corresponding to 4 x LD_{50}. Neutralization activity was expressed as ED_{50} defined as the amount of anti-venom (mg of protein) needed to reduce by 50% the lethal potency of the venom.

Results

Antigen purification

Sephadex G-75 chromatography column was an efficient step in the antigen purification. Results from a typical purification of PLA_2 are shown in Fig. 1. The homogeneity of the purified PLA_2 was examined by SDS-PAGE and by immunoprecipitation analysis. The antigen showed a single band of 16 kDa by SDS-PAGE method (Fig. 2) and gave a single precipitation line in the Ouchterlony method when the antigen was tested against ACS (Fig. 3).

Antibody production

Antibody production during the immunization process was monitored by double immunodiffusion until strong immunoprecipitin bands were consistently obtained (Fig. 4). Rabbits were bled and antibody serum titers were determined by ELISA. Fig. 5 shows the reactivity between PLA_2 and serial dilutions of the anti-sera raised in rabbits, and with ACS. The endpoint dilution of the anti-sera raised in rabbits was greater than for ACS, 1/218 700 and 1/8100, respectively.

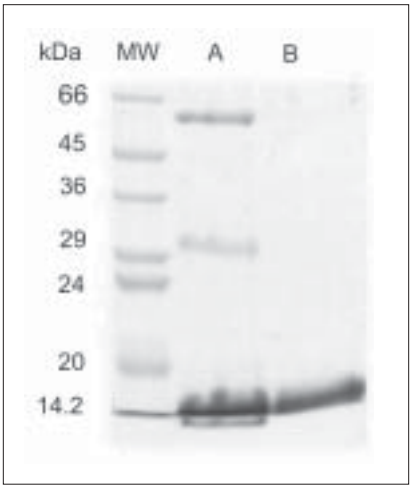

Fig. 2.– SDS-PAGE of the purified PLA_2. Electrophoresis was performed on 15% of polyacrylamide gel in SDS-PAGE at 200 V for 45 minutes. The gel was stained with 0.1% Coomasie blue R-250 (in 40% methanol and 12% Acetic acid) and distained with 10% acetic acid. (A) *C.d.t.* Venom (1 $mg.ml^{-1}$). (B) PLA_2 (2 $mg.ml^{-1}$).

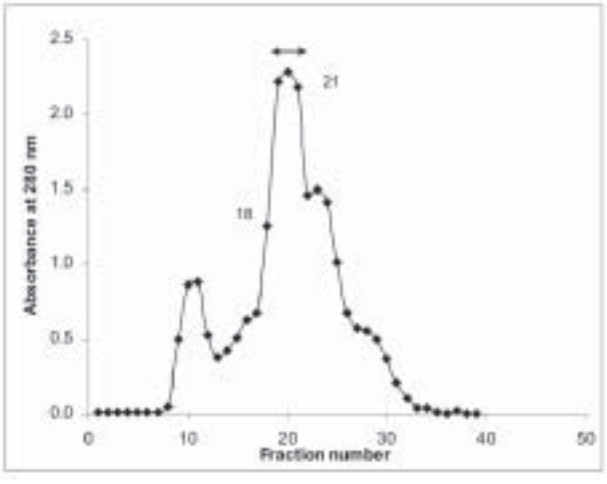

Fig. 1.– Elution profile of *Crotalus durissus terrificus* venom fractionated on Sephadex G-75. Venom (0.04 $g.ml^{-1}$, 0.1 ml) was applied to a column (1 x 30 cm) of Sephadex G-75 pre-equilibrated and eluted with glycine buffer 20 mM, 150 mM NaCl, pH 1.9. Double arrow showed fractions with PLA_2 activity.

Fig. 3.– Inmunodifussion of PLA_2 against ACS. 1) ACS, 2) Purified PLA_2 (1 $mg.ml^{-1}$), 3) whole venom (4 $mg.ml^{-1}$) and 4) whole venom (1 $mg.ml^{-1}$).

Fig. 4.– Ouchterlony double diffusion test. Immunodifussion of PLA$_2$ (1mg.ml^{-1}) (3) against anti-sera raised in rabbits (2) and IgG anti-PLA$_2$ (1).

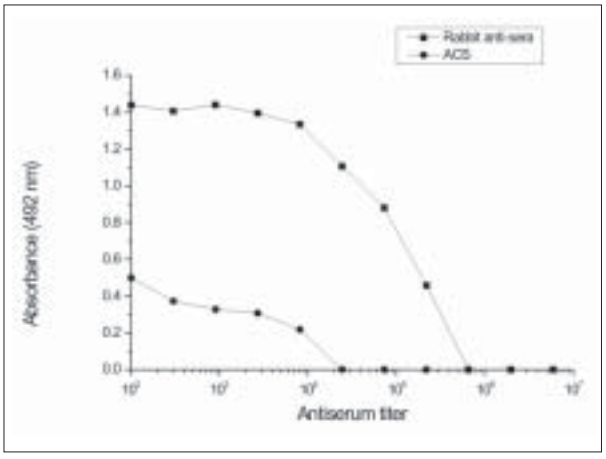

Fig. 5.– ELISA. Reactivity of anti-sera raised in rabbits and ACS with PLA$_2$ from *C.d.t.*

Immunoblotting

Figure 6 shows immunoblots of PLA$_2$ and whole venom detected with anti-sera raised in rabbit (diluted 1:1 000) IgG anti-PLA$_2$ (0.1 mg.ml^{-1}), and with ACS (diluted 1:2 000). All of the anti-sera recognized PLA$_2$ from *C.d.t.* venom.

Hemolytic activity neutralization

ED$_{50}$ for ACS and IgG anti-PLA$_2$ were calculated according to the amount of anti-venom (ACS or IgG anti-PLA$_2$)

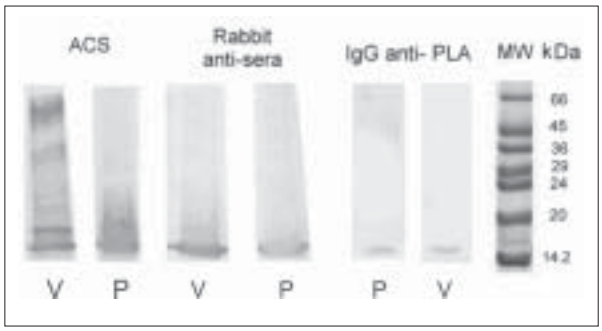

Fig. 6.– Immunoblotting. Blotting shows the reactivity of anti-sera raised in rabbits, ACS and IgG anti-PLA$_2$ with whole venom (V) and PLA$_2$ from *C.d.t.* (P).

TABLE 1.– *Comparison of the neutralization of the PLA$_2$ activity and lethal potency in* C.d.t. *venom with ACS or IgG anti-PLA$_2$*

	ED$_{50}$ ACS	ED$_{50}$ IgG anti-PLA$_2$
PLA$_2$ activity	3.1 ± 0.3	2.1 ± 0.4
Lethal potency	2.8 ± 0.3	1.8 ± 0.2

Results are given as means ± SD, n = 4

that reduce 50% of hemolytic activity. The results showed in Table 1 demonstrated that minor amounts of IgG anti-PLA$_2$ were needed to neutralize 1 HD (100 µg) of *C.d.t.* venom.

Lethal activity neutralization

The values obtained for ACS and IgG anti-PLA$_2$ in protecting against lethality in mice are shown in Table 1. IgG anti-PLA$_2$ proof to be better in neutralizing lethal activity.

Discussion

The methodology employed to purify PLA$_2$ consisted in only one step, a gel filtration chromatography at extremely acid conditions thus obtaining the enzyme in a high purity grade. This resulted in a simple method compared to Landucci et al.[5] technique in which two steps were applied, a gel filtration chromatography at pH 8, followed by an ion exchange chromatography. The molecular weight of the isolated PLA$_2$ proved to be in agreement with that reported by others[17, 18]. The SDS–PAGE analysis and the single precipitation line exhibited by immnunodiffusion analysis showed that the isolated PLA$_2$ was not contaminated either by crotapotin or other proteins present in

whole venom. Immunoblotting test confirmed the purity of the antigen when it was revealed with ACS.

The high titer obtained by ELISA confirmed the excellent reactivity of the anti-sera raised in rabbits to PLA_2, whereas ACS was demonstrated to be about 16-fold less reactive to this protein.

In order to improve the neutralization capacity of the antibodies, per mg of protein, specific antibodies, IgG anti-PLA_2 were purified. These antibodies neutralized the hemolytic activity and the lethal potency of the whole venom. ACS also efficiently neutralized the whole venom, but major quantities of proteins were required.

The greater neutralizing capacity of IgG anti-PLA_2 compared to ACS could be due that ACS is often semipurified globulin-rich preparation containing non-IgG proteins and aggregates[11, 19]. However, the electrophoretical test of ACS demonstrated that it does not contain those proteins (data not shown).

These results demonstrate that it is possible to obtain anti-venom made by specific antibodies with a high level of protection against the lethal component of the *C.d.t*. venom, and/or the inclusion of these antibodies as a supplement in heterologous anti-venoms. Further physiological and histopathological studies will be conducted in order to evaluate possible sequels induced by this type of treatment.

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9. Sólo aquellos que ceden consiguen. Cada fenómeno de la naturaleza no es más que una transacción de elementos. No se te ha hecho tal como eres para que te plantes como una mula. Entre la terquedad de Balaam y la de su burra, se interpuso el ángel; entonces transó la burra, y habló. De otra manera todavía estaría azotándola su amo. He ahí una montura superior a su jinete.

Almafuerte (Pedro Bonifacio Palacios) (1854-1917)

Al azar de las ideas. En: *Prosa y poesía de Almafuerte.* Selección y prólogo de Jorge Luis Borges. Buenos Aires: Eudeba, 1962, p 72