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Cross-neutralization of the coagulant activity of *Crotalus durissus terrificus* venom from the northeast of Argentina by bivalent bothropic antivenom

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Abstract: Cross-neutralization of *Crotalus durissus terrificus* venom coagulant activity was tested using bivalent horse antivenom against *Bothrops alternatus* and *Bothrops diporus* venoms. Our *in vitro* and *in vivo* experiments showed that bothropic antivenom neutralizes the thrombin-like activity of crotalic snake venom and this cross-reaction was demonstrated by immunoassays either with whole venom or a purified thrombin-like enzyme. These results suggest common antigenic properties and, consequently, similar molecular structure among venom thrombin-like enzymes. Besides, they provide information that could be further used in the development of new antivenom formulations.

Key words: thrombin-like enzyme, snake venom, antivenoms, immunological cross-reaction.

INTRODUCTION

Snake venoms, particularly those belonging to Viperidae family, often induce disorders in the blood coagulation system, which represents a serious complication in snakebites (1, 2). Venom proteases, which are generally classified structurally as serine proteases and metalloproteinases, play a crucial role (3-5). Several snake venom proteases may activate blood coagulation factors such as factor V or X, or act as prothrombin convertases or as thrombin-like enzymes (TLE) (6). These latter have a direct action on fibrinogen (thrombin like activity); they transform fibrinogen into fibrin gel. Since TLE do not activate factor XIII, the resulting fibrin clot is not cross-linked, hence it is easily degraded by the fibrinolytic system and removed from circulation by mononuclear phagocytes causing acute defibrinogenating effects (7).

Crotalus durissus terrificus (*C.d.t.*) venom may cause complete and long lasting incoagulability of blood in patients, due to the hypofibrinogenemia or afibrinogenemia developed after the bite (8). Besides the substitution therapy with plasma or plasma derived products, the use of antivenoms is the only effective treatment to this disturbance of hemostasis (9). On the other hand, several TLE have been isolated from *Bothrops* venom, and they are endowed with similar toxic activities described for the crotalic TLE (cTLE).

Numerous authors evaluated cross-reactions against toxins and antivenoms, and found interesting results. Immunochemical investigations performed with antibodies raised against the phospholipase A_2 (PLA_2) neurotoxin agkistrodotoxin (AGTX), from *Agkistrodon blomhoffii brevicaudus* venom, and against the PLA_2 subunit of crotoxin, from *C.d.t.* venom ($cPLA_2$), indicated that they share some structural similarities. The majority of AGTX antigenic determinants are present on $cPLA_2$ and

on PLA_{2s} from Viperidae venoms. Additionally, some of these determinants are involved in the neutralization of lethal potency and in the inhibition of enzymatic activity of AGTX and crotoxin (10).

On the other hand, Beghini *et al.* (11) demonstrated that it was possible to neutralize the neurotoxicity of *C.d.t.* and *Bothrops jararacussu* venoms and their major toxins, cdt-crotoxin and bothropstoxin-I respectively, with rabbit anti-sera produced against crotoxin and phospholipase A_2 from *C. durissus cascavela* venom.

A previous work of our laboratory demonstrated a cross-reaction among bothropic PLA_2 and IgG raised against $cPLA_2$ (12). We showed that although an immunological cross-reaction exists, specific anti-$cPLA_2$ antibodies do not have the appropriated pharmacological effect required for a complete neutralization of the PLA_2 activity of whole bothropic venoms. These data corroborate the finding by Chippaux and Goyffon (13) that a cross-reaction does not necessarily means cross-protection.

In view of the similarities among Viperidae venoms and the possible presence of similar antigenic determinants between crotalic and bothropic toxins, in this work, the immunological cross-reactions between these venoms and their thrombin-like enzymes were studied. Therefore, we tested the neutralization capacity of the bivalent antivenom produced for *Bothrops alternatus* (*B.a.*) and *Bothrops diporus* (*B.d.*) bites – both species are responsible for most bothropic envenomations in northeastern Argentina – against *C.d.t.* venom. In order to further demonstrate that this cross-neutralization exits, a thrombin-like serine proteinase from *C.d.t.* was isolated and two immune and coagulant activity neutralization tests were assayed.

MATERIALS AND METHODS

Venoms and Antivenoms

Desiccated *C.d.t.* venom and bothropic venoms were obtained from adult specimens from the serpentarium of Corrientes city. Horse antivenoms were obtained after eleven injections of *B.a.* or *B.d.* venom per animal, performed over a period of eight months. Complete Freund's adjuvant (Sigma, USA) was used in the first injection and booster inoculations were prepared with incomplete Freund's adjuvant (Sigma, USA). The antibody levels in the sera were monitored by ELISA. Blood samples were collected from the jugular vein, sera were subsequently separated by centrifugation and antibodies were purified by affinity chromatography (HiTrap Protein G HP 1 mL, Amersham Biosciences, Sweden) in a FPLC System, according to the manufacturer's instructions to obtain IgG fraction. Equal amount of antibodies from each antisera (IgG anti *B.a.* venom or IgG anti *B.d.* venom) were mixed and the reactivity and the specificity of the mixture (IgG_{av}) were tested by ELISA and dot blotting.

ELISA Assay

Briefly, microtiter plates (96 wells) were coated with 100 µL of *C.d.t., B.a., B.d.* venoms *or* TLE (5 $\mu g.mL^{-1}$) in PBS for one hour at 37°C. The plates were washed and processed as described by Rodríguez *et al.* (14). Absorbance was read at 490 nm with a Multiskan® EX (Thermo Scientific, USA) multiwell plate reader.

Dot Blotting Tests

In order to provide highly specific results a dot blot test was performed. Antigens (bothropic venoms, *C.d.t.* venom and cTLE) were diluted to different concentrations, in a 20 mM Tris 500 mM NaCl buffer (TBS), pH 7.2. Different amount of antigen samples (3 to 12 µg) were pipetted into each dot in a vertical row of a nitrocellulose strip (Biorad, USA). The membranes were dried at room temperature for two hours. After that, the strips were washed once with TBS containing 0.1% (v/v) Tween 20 (TBS-Tween 20) and blocked with bovine serum albumin (1% in TBS-Tween 20) for one hour at 37°C, on plates on a shaking platform. Then, strips were washed once with TBS-Tween 20 and immediately incubated with IgG_{av} for one hour at 37°C. Bound antibodies were detected with goat anti-horse IgG peroxidase conjugate (Sigma, USA; 1:1000 in TBS) incubating the strips in the same conditions. Finally, blots were washed, developed with 4-chloro-1-naphthol (Sigma, USA; 0.03% in 0.05 M Tris-HCl, pH 7.6, containing 0.03% H_2O_2/OPD) and documented. Horse pre-immune serum was employed as negative control.

cTLE Purification and TLE Activity

A crotalic thrombin-like enzyme (cTLE) was purified by a two-step chromatographic protocol as previously described (15, 16). Briefly, venom

samples of 25 mg, obtained from adult specimens, were applied to a Sephadex G-75 (100 x 1 cm) column pre-equilibrated with 20 mM glycine, 150 mM NaCl buffer, pH 1.9. Fractions with high coagulant activity were pooled, concentrated and a cTLE was purified with a Benzamidine-Sepharose Fast Flow affinity chromatography column (10 x 1 cm; Amersham Biosciences, Sweden), previously equilibrated with 0.05 M Tris 0.4 M NaCl buffer. After elution of the unbound fraction, 0.1 M sodium acetate 0.3 M NaCl pH 3.0 buffer was applied to the column and the absorbance of the eluting material was monitored at 280 nm.

In Vitro TLE Activity Neutralization Tests

Neutralization of thrombin-like activity was tested using a Wiener Lab Fibrintimer 2® coagulometer (Germany). Different amount of venoms or cTLE (10 to 320 µg) were incubated with IgG_{av} (35 mg/mL) for 30 minutes at 37°C and, then, 75 µL of the sample was added to 75 µL of human fibrinogen (4 mg/mL; Sigma, USA) or human plasma. The mixture of venom and IgG_{av} (V-IgG_{av}) was incubated at 37°C and the time until clot formation was measured ($T_{v\text{-}IgG}$). Venom solutions incubated with PBS were used as control samples (T_V). Complete inhibition was assumed when no clot formation occurred within ten minutes and zero inhibition when the clotting time was identical to that of the control sample (T_V). Percentage of neutralization was determinate using the ratio:

$$\% \text{ Neutralization} = \left(\frac{1 - T_v}{T_{v\text{-IgG}}}\right) \times 100$$

In Vivo Fibrinogen Consumption Neutralization Tests

Six groups of four mice each, weighing 18 to 22 g, were intraperitoneally (IP) injected with 0.5 µg/g of purified cTLE or cTLE preincubated with the amount of IgG_{av} previously determined for completel neutralization of enzyme activity. Animals of the control group were injected with PBS. After the determined time of exposure (0, 1, 3, 6, 9 and 24 hours), animals were anesthetized and blood was collected from the cava vein and, then, trisodium citrate was added to prevent coagulation. Platelet-poor plasma was obtained after centrifugation of samples at 2500 rpm at room temperature for ten minutes. Fibrinogen content was determined on each sample by triplicate using Wiener Lab Fibrinogen® kit (Argentina). Fibrinogen values were expressed in percentages assuming as 100% the fibrinogen level at initial time.

RESULTS

ELISA and Dot Blotting Tests

The reactivity determined by ELISA of IgG_{av} against bothropic venoms is shown in Figure 1 – A. As expected, the greatest reactivity was observed with *B.a.* and *B.d.* venoms, since they were used in the immunization process. On the other hand, *C.d.t.* venom and cTLE revealed similar behavior with absorbance values slightly over 50% of those

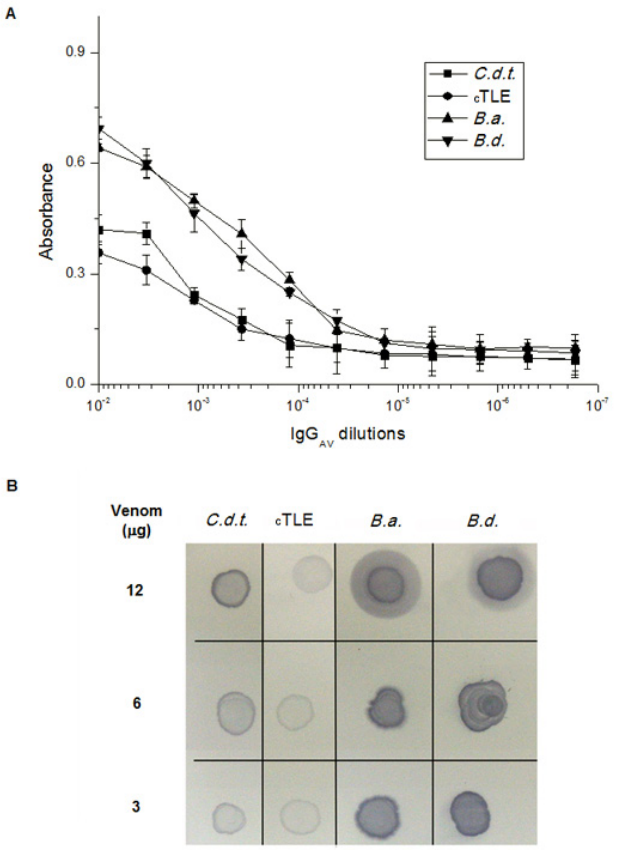

Figure 1. (**A**) Reactivity of IgG_{av} measured by ELISA, with crotalic and bothropic venoms, and cTLE. (**B**) Dot blot analysis of whole bothropic and crotalic venoms and purified cTLE with IgG_{av} antibodies. Control blots were carried out in the same conditions with PBS and did not show any spots. *C.d.t.*: *Crotalus durissus terrificus*; cTLE: crotalic thrombin-like enzyme; *B.a.*: *Bothrops alternates*; *B.d.*: *Bothrops diporus*.

obtained for bothropic venoms. The specificity of IgG_{av} was demonstrated with dot blot test (Figure 1 – B). It showed that IgG_{av} cross-reacted with *C.d.t.* venom and its cTLE, strong spots were noted in each case, even with 3 µg of protein.

In Vitro Neutralization Tests

IgG_{av} was able to neutralize the coagulant activity of bothropic venoms, and cross-neutralize crotalic venoms and cTLE. Figure 2 – A shows the neutralization of coagulant activity of bothropic and crotalic venoms by IgG_{av} on human fibrinogen. The IgG_{av}:venom ratio required to efficiently neutralize the bothropic coagulant activity was 26.25 µg IgG_{av}/µg of venom. At the same IgG_{av}:venom ratio, it was observed 80% of *C.d.t.* venom cross-neutralization. However, it was necessary a three-fold increase of IgG_{av}:venom ratio to show 100% of *C.d.t.* venom neutralization.

In order to reproduce *in vitro* a more realistic scenario, similar results were obtained when the neutralization of plasma coagulant activity was tested with IgG_{av}. Figure 2 – B shows that the IgG_{av}:venom ratio needed to neutralize the activity of venoms is the same, and both bothropic venoms were efficiently neutralized at this ratio (100%).

The results presented in Figure 3 demonstrate that cTLE serine proteinases from *C.d.t.* venom

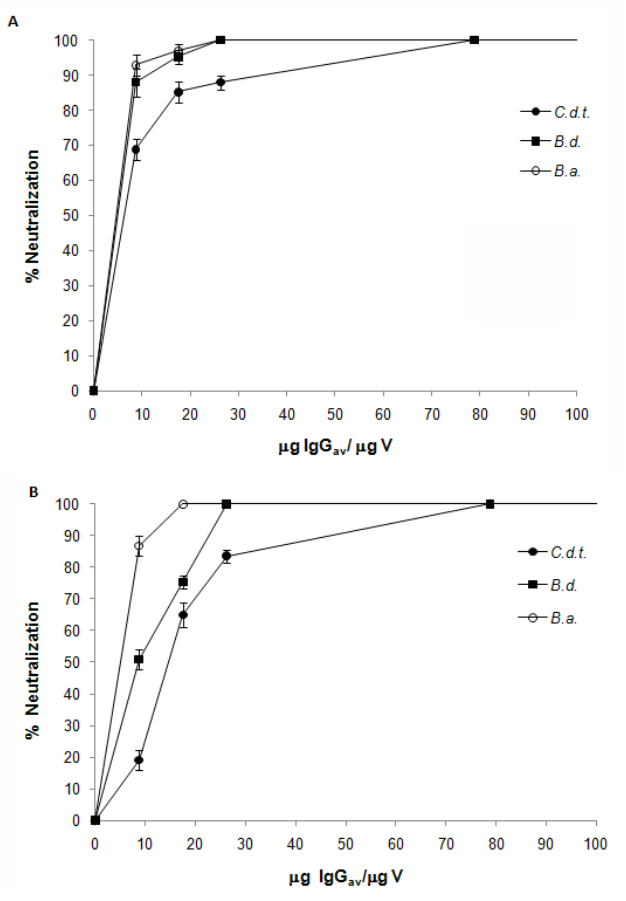

Figure 2. Neutralization of coagulant activity of bothropic and crotalic venoms by bothropic antivenom. (**A**) Micrograms of IgG_{av} per microgram of venom for the neutralization of fibrinogen coagulant activity. (**B**) Micrograms of IgG_{av} per microgram of venom required to neutralize plasma coagulant activity. Results are presented as mean ± S.D. (n = 4). *C.d.t.*: *Crotalus durissus terrificus*; *B.a.*: *Bothrops alternates*; *B.d.*: *Bothrops diporus*; V: venom.

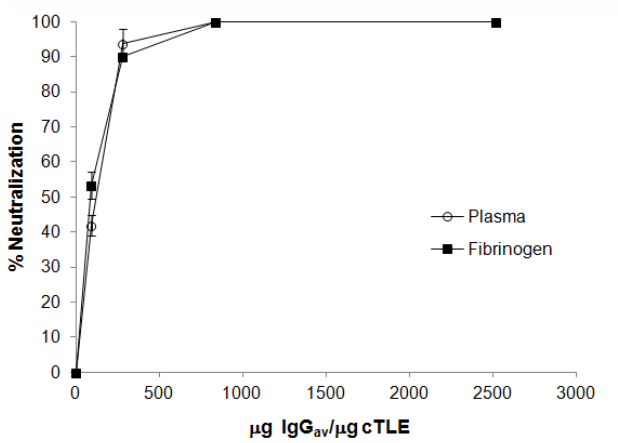

Figure 3. Neutralization of the coagulant activity of cTLE by IgG_{av}. Results are presented as mean ± S.D. (n = 4). cTLE: crotalic thrombin-like enzyme.

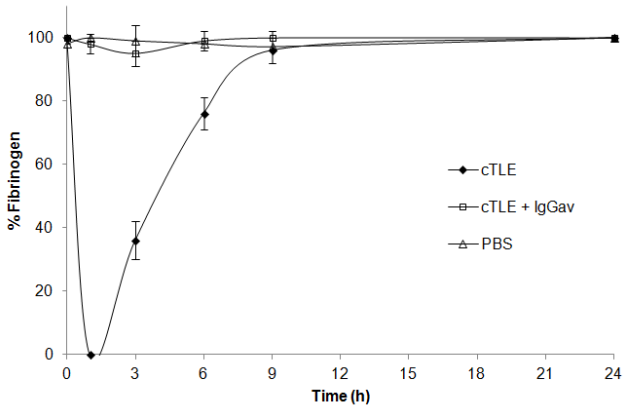

Figure 4. Neutralization of *in vivo* fibrinogen consumption of cTLE by IgG_{av}. Results are presented as mean ± S.D. (n = 4). cTLE: crotalic thrombin-like enzyme.

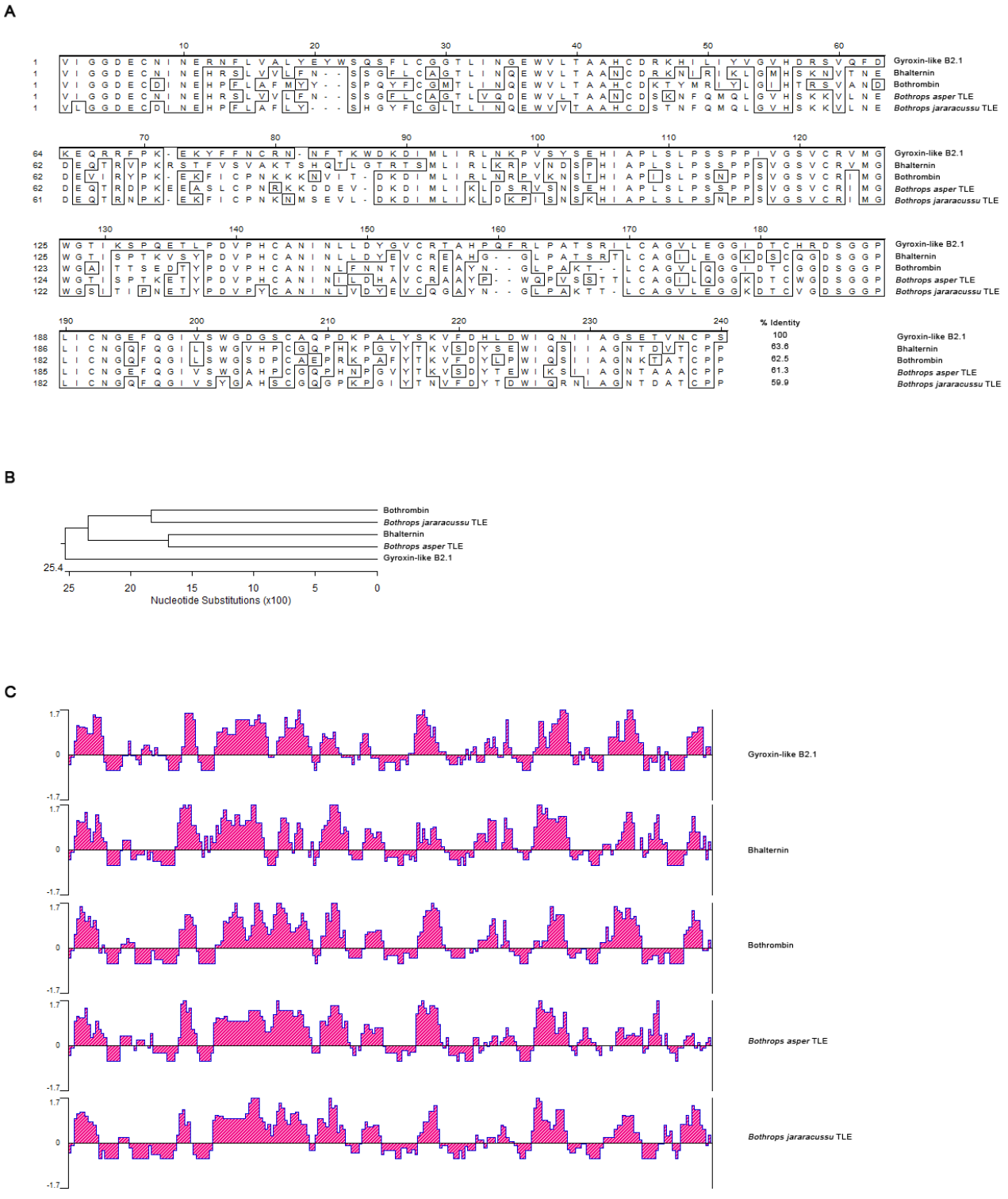

Figure 5. (**A**) Alignment of the amino acid sequence of several bothropic TLEs with gyroxin-like B2.1 from *Crotalus durissus terrificus*. (**B**) Phylogenetic tree constructed with four bothropic thrombin-like enzymes and a crotalic thrombin-like enzyme (gyroxin-like B2.1). (**C**) Antigenic profiles calculated with Jameson and Wolf (19) algorithm from the linear amino acid sequences of bothropic and crotalic TLEs.

are cross-neutralized by IgG_{av}. In that sense, similar neutralization tests were developed with cTLE, purified from whole venom, on plasma and fibrinogen. Since the same amount of enzyme was employed instead of whole venom (10 to 320 µg), the high clotting activity of this sample required a 10.7-fold increase of IgG_{av}:TLE ratio to show 100% neutralization of enzymes, compared to IgG_{av}:venom ratio, needed to cross-neutralize *C.d.t.* venom.

In Vivo Neutralization Tests

The effect of cTLE neutralized by IgG_{av} was examined *in vivo*. Animals injected with cTLE showed rapid drop of plasma fibrinogen levels during the first hour, to undetectable values, then reaching its normal levels 10 hours after injection. On the other hand, cTLE (10 µg) preincubated with IgG_{av} (according to the relation found in the *in vitro* assay), resulted in minimal decrease of the mouse plasma fibrinogen, and no significance differences was detected when these results were compared with those obtained in the control group (Figure 4).

Theoretical Structural Analysis

In order to explain the experimental results found, we have analyzed several sequenced toxins homologous to those used in the neutralization experiments of this work. Figure 5 details the structural aspects studied with MegAlignTM software ver. 5.01 (DNASTAR, USA).

DISCUSSION

In vitro evidence of immunological cross-reactivity is reported for many venoms and antivenoms as a way of detecting similarities among toxins and potential uses of antivenoms. Such cross-reactions have been studied by other authors using western blot and ELISA; however, they need to be individually verified with biological tests, according to the toxicological properties of venoms (13).

The present results indicated that IgG_{av} was capable to cross-react with *C.d.t.* venom since sharp spots were obtained in a dot blot test and the ELISA test confirmed its reactivity.

Moreover, antibodies produced against bothropic venoms were able to block the coagulant activity of *C.d.t.* venom. These results are in opposition to those observed by de Roodt *et al.* (17), who stated that the coagulant activity of *C.d.t.* was not affected by preincubation with bothropic antivenoms. Furthermore, our results are supported by immunoassays and biological neutralization tests carried out with the isolated enzyme.

The finding that antibodies against bothropic venoms recognize crotalic TLE reveals structural similarities among viperid thrombins in spite of the large differences between the two genera. In order to explain this experimentally observed phenomenon, we analyzed some structural studies in the literature, with the aim of correlating the degree of conservation and homology of the molecules that cross-react with IgG_{av}.

Some proteins, already sequenced and homologous to those studied here, were selected for analysis. Thus, gyroxin-like B2.1 (NCBI protein database accession number: Q58G94) – isolated by Brazilian authors from *C.d.t.* venom – and bhalternin (NCBI protein database accession number: P0CG03) – purified from *B. alternatus* venom from Brazilian specimens – were chosen (3). In addition, other three thrombins from venoms from other bothropic species – *B. asper* (NCBI protein database accession number: ABB76280), bothrombin from *B. jararaca* (NCBI Protein Database accession: P81661) and *B. jararacussu* (NCBI protein database accession number: ABC24687) – were included (18).

The homology analysis of protein sequences developed with MegAlign™ software ver. 5.01 (DNASTAR, USA) allowed us to detect over 63% of identity between gyroxin like B2.1 and bhalternin, and slightly lower percentages with other enzymes (Figure 5 – A). This would predict the existence of sequential epitopes shared. Figure 5 – B illustrates that gyroxin is the most phylogenetically separated compared with the other bothropic toxins analyzed. These observations corroborate our immunological cross-reaction tests between the crotalic venom and bothropic antivenom.

Furthermore, since antigenic sites are located within surface-exposed regions of a protein, Protean™ software ver 5.01 (DNASTAR, USA) was employed to generate accurate antigenic profiles from the linear amino acid sequences with Jameson and Wolf algorithm (19). Figure 5 – C shows similar topological profiles between gyroxin and bhalternin that would explain potential conformational antigenic

determinants shared, similar to those detected experimentally with IgG_{av}. The other bothropic toxins also present a similar antigenic profile and it would not be surprising to find that they also show immunologic cross-reactivity; however, individual verification is required.

Clotting activity neutralization tests on plasma and on fibrinogen, of whole venom and cTLE, revealed that a higher amount of antibodies (approximately 10-fold) is required to neutralize 100% of the enzyme activity compared to that needed to neutralize whole venom. This is expected since whole venom is composed of a set of proteins among which thrombin-like enzymes, with serine protease structures, represent only 8% of it, as reported by Calvete *et al.* (20) in a venomic study of *Crotalus* species.

Additionally, an *in vivo* incoagulability assay was performed in order to provide further evidence of this immunological cross-reaction and render the work more conclusive. In a previous work by Maruñak *et al.* (15), the effects of cTLE and whole venom on mice plasmatic fibrinogen levels were characterized. It showed that fibrinogen concentrations decreased rapidly to incoagulability during the first hour after cTLE injection, and then returned to its normal levels ten hours later (15). In the present study, we demonstrated that it is possible to neutralize this incoagulability effect of cTLE preincubating with IgG_{av}.

Current research on antiophidic therapy is focused on the production of new antivenoms with reduced protein content and high neutralization capacity. This can be achieved using either specific antibodies with the ability to inhibit the most toxic proteins from different venoms or antibodies that cross-react with them. Evidences of immunological cross-reactions between crotalic venoms and bothropic antivenoms presented herein reinforce the use of polyvalent antivenoms, which could have practical applications in the development of future new antitoxin formulations.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FINANCIAL SOURCE

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics and Biosafety Committee of the School of Veterinary Science, Northeast National University, Argentina.

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