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Short communication

Ability of horse anti-*Tityus discrepans* F(ab')$_2$ ELISA assay to recognize *Tityus discrepans* venom toxins

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ABSTRACT

Anti-*Tityus discrepans* F(ab')$_2$ ELISA recognition of *T. discrepans* toxins was measured with regression analysis and its slope called ELISA recognition value (ERv). Fractions containing toxins affecting mammal macrophages or Na^+-channels have Ervs >19. Toxins affecting potassium channels or insect Na_V channels have ERvs <10. Fractions including curarizing or antineoplasic peptides had ERvs <1. Erv increases in proportion to mammalian toxin toxicity rather than to toxin molecular mass.

1. Introduction

The severity of clinical manifestations and complications during scorpion envenoming depend on venom blood concentration (D'Suze et al., 2003). During the last forty years, several enzyme-linked immunosorbent assays (ELISAs) have been developed to quantify venom in blood plasma or serum (Chávez-Olortegui et al., 1994; Coulter et al., 1980; D'Suze et al., 2003; Engvall and Perlmann, 1971; Krifi et al., 1998; De Rezende et al., 1996; Theatkston et al., 1977). A very accurate ELISA able to discriminate among scorpionism patients (D'Suze et al., 2003) was developed using immunopurified polyclonal antibodies against *Tityus discrepans* venom; this assay allowed to quantify venom in nanograms per milliliter concentrations. This ELISA has been used in both experimental and diagnostic assays, and enabled novel pharmacokinetic studies (Sevcik et al., 2004). However the question remains: Does this detection method recognizes equally well all venom components?, the answer to this question is addressed here.

2. Materials and methods

2.1. Venom source and extraction

Adult *T. discrepans* scorpions were collected around Caracas (Venezuela) and kept in plastic boxes with water and food *ad libitum*. Adult specimens (≈200) were anesthetized with CO_2 and milked for venom by electrical stimulation once a month during 3 months. Venom was dissolved in double distilled water and centrifuged at 15,000 *g* for 15 min at 4 °C. Protein content was estimated based on absorbance at 280 nm, assuming 1 mg/mL protein represents 1 unit of absorbance per unit path length. The supernatant was freeze dried using a Savant Speed-Vac dryer (SC110, New York) and stored at −80 °C until used.

2.2. Venom components isolation

Venom was fractionated through an analytical reversed phase C18 column (250 × 10 mm, Vydac, Hesperia, CA)

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coupled on a high-performance liquid chromatograph (HPLC) with a photodiode array detector (Water Corp. 2695, 2996). Elution of venom components was performed at a flow rate of 1 mL/min using a linear gradient from solution A [0.12% trifluoroacetic acid (TFA) in water] to 60% solution B [0.10% TFA in acetonitrile (ACN)] in 60 min and detected at a wavelength of 230 nm. Fractions were manually collected, freeze-dried and stored at −80 °C until used.

2.3. Enzyme-linked immunosorbent assays

The ELISA used to measure different venom fractions at different concentration was developed following the D'Suze et al. (2003) protocol with some modifications. Briefly, specific anti-*T. discrepans* $F(ab')_2$ antibodies (from Suero Antiescorpiónico™ antivenom, Centro de Biotecnología, UCV, Caracas, Venezuela) were purified by affinity chromatography on a Sepharose® 4B CNBr-activated column to which 10 mg of *T. discrepans* venom was coupled. Venom diluted 1:1 in 50 mM Tris/HCl pH 8, was applied on the column. The bound fraction was eluted with 0.1 M acetic acid and collected as 500 mL aliquots containing 1 M Tris/HCl pH 8. Maxisorp (Nunc Inc, USA), plates were coated overnight at 4 °C with affinity-purified antibody (1 μg/well) diluted in 100 μL of 100 mM carbonate/bicarbonate buffer, pH 9.5. Then plates were washed 4 times with washing buffer (50 mM Tris/HCl pH 8, 150 mM NaCl and 0.05% tween 20). The remaining binding sites were blocked with 3% skim cow milk in washing buffer for 1 h at 37 °C (100 μL/well). After plates were washed 4 times with washing buffer. Samples of whole venom and isolated venom fractions in a concentration range from 0.45 to 166 ng/mL were dissolved in plasma. Tween 20 and NaCl were added to reach a concentration of 0.05% and 300 mM, respectively. Plates were incubated 1 h at 37 °C, its were washed 4 times with washing buffer and incubated again 1 h at 37 °C with 125 ng/mL anti-*Tityus* rabbit immunoglobulin G (IgG) produced and purified in our laboratory following same methods described above for anti-*T. discrepans* $F(ab')_2$ (D'Suze et al., 2003). Plates were washed and incubated 1 h at 37 °C (100 μL/well) with anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Zymed California, EEUU) diluted 1:5000 in washing buffer. After washing, 100 mL/well ABTS (Zymed, California USA) diluted 1:100 in citrate 0.1 M pH 4.2 containing 0.03% hydrogen peroxide were added and incubated 15 min at 25 °C protected from light, after this time the reaction was stopped with 25 mL/well of 2 N fluorhydric acid. Absorbances were read after 3 min at 405 nm with a Multiskan Spectrum spectrophotometer (ThermoLife Sciences, Basingstoke, UK).

2.4. Venom components detection

Whole venom and HPLC isolated venom fractions were assayed in triplicate by the sandwich ELISA test described above. Some fractions having small amounts of protein, as well as valleys between two peaks, were grouped. Samples were dissolved with plasma in presence of 0.05% tween 20 and 300 mM NaCl. Enzyme-linked immunosorbent assays were developed to determine samples detection in a concentration range from 0.45 to 166 ng/mL sample. Lineal regression analysis for each sample were made using Excell package linear regression tool (Microsoft Office software, Microsoft, Redmont WA). The slope of the curve represented the ELISA recognition value (Erv) for each sample.

2.5. Statistical analysis

The data were processed using nonparametric statistical procedures. Data are presented as medians and their 95% confidence interval, calculated with the procedure of Hodges and Lehmann. See Holander and Wolfe (1973) for details.

3. Results and discussion

T. discrepans venom is a mixture of low and high molecular mass (MW) proteins, some of them metallo- or serine protease-like enzymes, mucopolysaccharides, salts, lipids and amino acids, (Batista et al., 2006; Brazón et al., 2009, 2013, 2014). The chromatographic profile of this venom on a C18 reverse-phase HPLC column, revealed 40 fractions eluted from 3.8 to 52 min, which were numbered in elution sequence as F1 to F40 (Fig. 1A). Previous studies using MALDI-TOF and nano-ESI-ITMS demonstrated the presence of 205 components with molecular masses from 272 to 57,908 amu in these fractions (Batista et al., 2006). The molecular weight (MW) distribution in the venom, showed a high proportion of low-MW peptides. Peptides with MW < 5 kDa represented approximately 17% of whole venom, some of them able to block K^+ channels (Batista et al., 2006). Peptides with MW between 6 and 8 kDa matched around 23% of whole venom (Batista et al., 2006), this group of toxins are able to modulate the conductance and/or other properties of sodium channels (Forsyth et al., 2012; Peigneur et al., 2012). In the present work they eluted into 8 different peaks between 32.5 and 37.3 min (Fig. 1A) corresponding to 52% of the total area under the curve. The remaining 32 peaks matched 48% of the total area under the curve. Therefore, we can say that toxins acting on sodium channels are the most abundant peptides in concentration, but not necessarily in diversity. They are the responsible of most scorpionism symptoms.

T. discrepans venom components are recognized in varying degrees by polyclonal antibodies against this venom. The ELISA developed to quantify Td venom in plasma was used here to measure recognition differences among venom components. The recognition called here "ELISA recognition value (Erv)" was determined with the slope of lineal regression analysis performed from a graph where the abscissa corresponds venom concentration (ng/mL) and the ordinate represents absorbances measured at 405 nm. Detection curves of whole venom and isolated venom fractions had neat linear correlations, with correlation coefficients between 0.97 and 0.99 (Fig. 1B). The slope of each curve called ERv as indicated above is shown in Table 1. As was expected whole venom had the highest recognition with a value of 85. The ERvs of isolated fractions