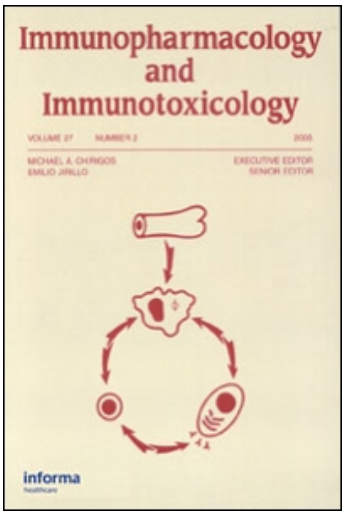

Immunopharmacology and Immunotoxicology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597257

Inhibition of the Hemorrhagic and Proteolytic Activities of Lansberg's Hognose Pit Viper (Porthidium lansbergii hutmanni) Venom by Opossum (Didelphis marsupialis) Serum: Isolation of Didelphis Marsupialis 0.15Dm Fraction on DEAE-Cellulose Chromatography

María E. Pineda [a]; María E. Girón [b]; Amalid Estrella [b]; Elda E. Sánchez [c]; Irma Aguilar [b]; Irma Fernandez [a]; Alba M. Vargas [a]; Héctor Scannone [a]; Alexis Rodríguez-Acosta [b]
[a] Pharmaceutical Research Laboratory of the Pharmacy Faculty, Central University of Venezuela, Caracas, Venezuela [b] Immunochemistry Section of the Tropical Medicine Institute, Central University of Venezuela, Caracas, Venezuela [c] Natural Toxins Research Center (NTRC), Texas A & M University-Kingsville, Kingsville, Texas, USA

First Published on: 17 September 2008

To cite this Article Pineda, María E., Girón, María E., Estrella, Amalid, Sánchez, Elda E., Aguilar, Irma, Fernandez, Irma, Vargas, Alba M., Scannone, Héctor and Rodríguez-Acosta, Alexis(2008)'Inhibition of the Hemorrhagic and Proteolytic Activities of Lansberg's Hognose Pit Viper (Porthidium lansbergii hutmanni) Venom by Opossum (Didelphis marsupialis) Serum: Isolation of Didelphis Marsupialis 0.15Dm Fraction on DEAE-Cellulose Chromatography',Immunopharmacology and Immunotoxicology,

To link to this Article: DOI: 10.1080/08923970802135849

URL: http://dx.doi.org/10.1080/08923970802135849

Immunopharmacology and Immunotoxicology (2008) i*First, 1–14*
Copyright © Informa UK, Ltd.
ISSN: 0892-3973 print / 1532-2513 online
DOI: 10.1080/08923970802135849

informa
healthcare

Inhibition of the Hemorrhagic and Proteolytic Activities of Lansberg's Hognose Pit Viper (*Porthidium lansbergii hutmanni*) Venom by Opossum (*Didelphis marsupialis*) Serum: Isolation of *Didelphis Marsupialis* 0.15Dm Fraction on DEAE-Cellulose Chromatography

María E. Pineda,[1] María E. Girón,[2] Amalid Estrella,[2] Elda E. Sánchez,[3] Irma Aguilar,[2] Irma Fernandez,[1] Alba M. Vargas,[1] Héctor Scannone,[1] and Alexis Rodríguez-Acosta[2]

[1]Pharmaceutical Research Laboratory of the Pharmacy Faculty, Central University of Venezuela, Caracas, Venezuela
[2]Immunochemistry Section of the Tropical Medicine Institute, Central University of Venezuela, Caracas, Venezuela
[3]Natural Toxins Research Center (NTRC), Texas A & M University-Kingsville, Kingsville, Texas, USA

Earlier studies have revealed the ability of sera from several mammals to neutralize the toxic effects of snake venom. The Venezuelan opossum (*Didelphis marsupialis*) is one that has been found to inhibit hemorrhagic and proteolytic activities of venoms from many species of snakes. In this article it is shown that the opossum sera and its 0.15DM fraction were able to completely neutralize both hemorrhagic and hydrolysis (proteolysis) of casein effects induced by venom of the Lansberg's hognose pit viper (*Porthidium lansbergii hutmanni*). We have used DEAE-cellulose ion exchange chromatography to collect

Address correspondence to Alexis Rodríguez-Acosta, M.D, Ph.D, Apartado 47423 Caracas 1041, Venezuela; E-mail: rodriguezacosta1946@yahoo.es

protein fractions from *D. marsupialis* sera which were able to defend mice from the lethal effects of *P.l. hutmanni* venom. The fractions separated were homogeneous by conventional electrophoresis using SDS-PAGE. The protein bands obtained contained molecular weights of ~ 6 to 220 kDa. These results revealed the presence of proteases inhibitors in the opossum sera fractions and the inhibition of venom activity by opossum sera suggesting a reciprocal adaptation at the molecular level.

Keywords *Didelphis marsupialis*, Hemorrhagic Activity, Opossum, *Porthidium lansbergii hutmanni*, Proteolytic Activity, Venom.

INTRODUCTION

The Lansberg's hognose pit viper (*Porthidium lansbergii hutmanni*) is responsible for an important number of snakebite accidents in Margarita Island, Venezuela. Its venom can induce acute renal failure and intense general hemorrhages.

The venoms of snakes, scorpions and spiders, but primarily from snakes, have a large quantity of proteins that influence hemostasis in different ways. Many of these proteins have enzymatic activities, such as phospholipases A_2 (PLA_2), serine proteinases, metalloproteinases and nucleotidases; while others like C-type lectins and disintegrins do not exhibit enzymatic activity. Enzymes like PLA_2s hydrolyze negatively charged phospholipids, which are cofactors of the prothrombinase complex, consequently with anti-coagulant activity, whereas others acting on the platelet membrane, hydrolyze phospholipids resulting in a discharge of platelet aggregating factors that induces platelet aggregation.[1]

A number of serine proteinases and metalloproteinases affect hemostasis, altering coagulation and specific factors in the blood coagulation cascade, and thus taking action on fibrinogenolysis and fibrinolysis.[2,3] These metalloproteinases from snake venom that alter hemostatic activities have an equal number of natural inhibitors, existing as plasma or muscle proteins of some mammals, such as opossums, squirrels, wild mice and skunks.[4] These natural inhibitors can be grouped as inhibitors of either phospholipase A_2 (antimyotoxic and antineurotoxic factors) or snake venom metalloproteinases.

These proteinase inhibitors have also been found in plasma of several snakes,[5] which act as mechanisms of natural or innate immunity. This kind of immunity is an extensively-based explanation of the actions of the evolutionarily preserved molecules and developments of the natural immune system, which must satisfy three requirements:

(a) they are present prior to foreign exposure;

(b) they are not enhanced by foreign exposure; and

(c) they do not differentiate between foreign antigens.[5]

This is a preliminary study exemplifying how opossum (*D. marsupialis*) sera and its fraction are able to inhibit the toxic venom activities of the

Lansberg's hognose pit viper[6] through their natural immunity. Thus, the purpose of this work was to confirm the occurrence of these molecules in *D. marsupialis* sera, which could signify a natural supplementary immune protection for the opossum against *Porthidium* venom. These natural inhibitors can accomplish the function of soluble ligands promptly accessible to inhibit the harmful effects of enzymatic molecules of the venom and could be used not only as therapeutic tools to treat ophidic accidents caused by *Porthidium* snakes, but as potential antagonist for metalloproteinase activities associated with many diseases.

MATERIALS AND METHODS

Reagents

Sephadex G-100 size exclusion (SE) high-performance liquid chromatography was from Sigma (Missouri, MO, USA). Molecular mass protein standards were obtained from Bio-Rad (Hercules, CA, USA). All other chemicals were reagent grade or equivalent.

Venom

Crude venom from *P. l. hutmanni* was extracted from specimens captured near Porlamar city, Margarita Island, Venezuela and maintained in the serpentarium of the Tropical Medicine Institute of the Universidad Central de Venezuela. Venom was collected over ice, frozen, lyophilized and maintained at – 80 ºC.

Animals

Outbreed male NIH Swiss albino mice weighing 18–22 g were used for all studies and obtained from the central animal house of the Instituto Nacional de Higiene "Rafael Rangel," Caracas, Venezuela. All the animals were maintained under strict ethical conditions according to international recommendations on animal welfare.[7]

Determination of Protein Concentration

The protein determination method followed the method of Lowry et al.[8] or by absorbance at 280 nm, standardized with bovine serum albumin.

Porthidium lansbergii hutmanni Venom Fractionation

Crude, lyophilized venom (400 mg by protein estimation) was reconstituted with 4 mL of ammonium acetate 50 mM buffer (pH 6.9) and exposed to a Sephadex G-100 column. Elution of protein was monitored at 280 nm. The eluting pinnacles of the venom peaks were tested for hemorrhagic activity. Peak 2 (P2Plh; 100 mg/1 mL) was selected for neutralization due to its high

hemorrhagic activity. This peak was dialyzed against water at 4ºC and lyophilized. Column operations were done at 4°C.

Sodium dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of *P. l. hutmanni* Venom and Venom Peak 2 (P2Plh)

SDS-PAGE of crude venom and its fraction P2Plh was carried out according to the Laemmli method,[9] using 12.5% gels under non-reducing conditions. Molecular mass markers (Bio-Rad, USA) were run in parallel and gels were stained with Coomassie blue R-250. The molecular mass was determined by Multi-Analyst TM/PC version 1.1 software (Bio-Rad).

Preparation of Immunoglobulin Free Opossum Sera

Blood from heart puncture from ten opossums was obtained and kept at 37ºC for 15 min to clot. Then sera were separated by centrifugation at 3000 g for 10 min at 5ºC. Resultant sera were diluted 1:2 with 0.9% NaCl. Saturated ammonium sulphate solution was added drop by drop to the sera until a final concentration of 36% (v/v) was reached. After 45 min the solution was centrifuged at 4ºC and the supernatants were recovered and dialyzed twice against PBS 0.005 M NaCL, pH 7.5 and frozen at –30º C until use.

DEAE-Cellulose Chromatography of Immunoglobulin Free Opossum Sera

Immunoglobulin free opossum sera were bound to a DEAE-cellulose column at 4°C. The proteins were eluted with 0.005 M PBS NaCl, pH 7.5 through a stepwise NaCl gradient using increased ionic strengths (0.05 M, 0.1 M, 0.15 M and 0.2 M). Fractions were concentrated by vacuum dialysis, dispensed in vials and stored at –30ºC.

Sodium dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of *D. marsupialis* Sera and its Fraction 0.15Dm

SDS-PAGE of complete sera and its fraction 0.15Dm was carried out according to the Laemmli method,[9] using 12.5% gels under non-reducing conditions. Molecular mass markers (Bio-Rad, Hercules, CA, USA) were run in parallel and gels were stained with Coomassie blue R-250. The molecular mass was determined by Multi-Analyst TM/PC version 1.1 software (Bio-Rad).

Lethal Activity (LD_{50})

The lethality of *P. l. hutmani* P2Plh fraction was determined by the Spearman-Karber method.[10] Five mice per dose weighing 18–22 g were

intravenously injected with 200 μL of the serially diluted hemorrhagic fraction (P2Plh) ranging from 2 to 200 μg. Deaths were recorded during a 48 h period. Mice were observed up to 48 h after injection.

Effective Dose (ED_{50}) of *D. marsupialis* Sera and its Fraction 0.15Dm

The effective dose of *D. marsupialis* sera and fraction 0.15Dm was determined by the Spearman-Karber method[10] Five mice per dose weighing 18–22 g were intravenously injected with 200 μL of serially diluted serum or fraction 0.15Dm containing 3 LD_{50} of *P. l. hutmanni* P2Plh fraction. Deaths were recorded during a 48 h period post injection.

Minimal Hemorrhagic Dose (MHD)

The method of Omori-Satoh et al. [11] was used to determine the minimal hemorrhagic dose (MHD). A series of eight dilutions were prepared with the hemorrhagic fraction (P2Plh), of which 0.1 mL of each dilution was injected intracutaneously into the abdominal skin of three male NIH Swiss albino mice. The mice were sacrificed with CO_2 after 8 h, and the internal skin surface was observed for hemorrhage. A caliper was used to measure the hemorrhagic diameter on the skin and the MHD determined. The MHD is defined as the amount of venom protein that causes a 10 mm hemorrhagic spot.

Neutralization of Hemorrhagic Activity by 0.15Dm

Inhibition of hemorrhagic activity was measured by incubating *P. l. hutmanni* P2Plh fraction (15 μg) for 30 min at 37ºC in PBS 0.005 M NaCL (pH 7.5) containing the *D. marsupialis* fraction eluting at 0.15 M buffer (0.15Dm). The sample was then injected intradermally into the abdominal skin of three male NIH Swiss albino mice. Aliquots of 100 μL containing the MHD (15 μg) of P2Plh were also injected into three male NIH Swiss albino mice as positive controls. Hemorrhagic activity was determined as described above. The antihemorrhagic activity was measured by the reduction of the hemorrhagic spot.

Caseinolytic Activity

A procedure carried out by Lomonte and Gutierrez[12] was used to determine caseinolytic activity. P2Plh (5–40 mg) and crude venom (10–80 mg) were incubated with 1 mL of the casein substrate (1% in 0.1 M Tris-HCl, pH 8.0) for 20 min at 37ºC. The reaction was stopped by adding 3.0 mL of 5% trichloroacetic acid. After 30 min, tubes were centrifuged at 3000*g* and the absorbances of the supernatants were measured at 280 nm. Control tubes containing substrates without P2Plh were included, and the absorbances of these samples were deducted from the experimental values.

Proteolytic activity was expressed in units/mg protein. The specific activity was expressed in U/mg protein, and calculated as follows: change in absorbance in 1 h divided by mg of protein used.

Inhibition of Caseinolytic Activity by 0.15Dm Fraction

Inhibition of caseinolytic activity was determined by incubating *P. l. hutmanni* venom or P2Plh venom fraction with 0.15Dm fraction for 30 min at 37 ºC. The mixture was then incubated with 1 mL of casein substrate for 20 min at 35°C. After incubation, proteolytic activity was tested on casein and calculated as described above. Control samples had the same amount of P2Plh without 0.15Dm.

RESULTS

Protein Concentration of *P. l. hutmanni* Crude Venom, Peaks 1 and 2 and *D.marsupialis* Protein Sera Concentration

Crude venom presented 90 % of proteins related with the total weight of the lyophilzed sample. In contrast, peaks 1 and 2 had 71.3 and 72.0 % of proteins, respectively. Complete *D. marsupialis* protein sera concentration was 47. 40 mg/mL.

Porthidium lansbergii hutmanni Venom Fractionation

Figure 1 shows four well-defined venom peaks obtained with a Sephadex G-100 chromatography column. The total elution volume from each peak was measured, and then the total sample recovery was based on the optical density readings at 280 nm (1 absorbance unit is equivalent to 1mg/mL of total protein). The approximate protein recovery was of 43 mg (43%) for Peak

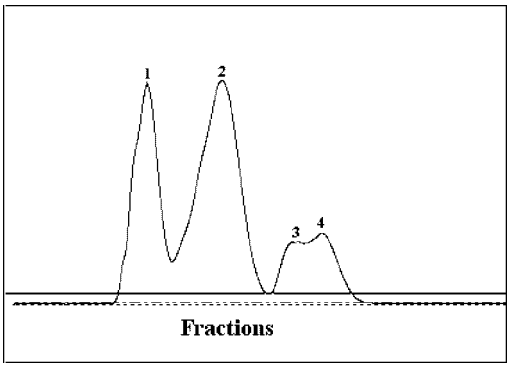

Figure 1: *Porthidium lansbergii hutmanni* crude venom elution profile obtained by Sephadex G-100 molecular exclusion chromatography column using ammonium acetate 50 mM buffer (pH 6.9). Elution of protein was monitored at 280 nm.

1 and 40 mg (40%) for Peak 2, in comparison with the total crude venom initially processed (approximately 100 mg). Each Peak was immediately dialyzed, lyophilized and stored at 4ºC until its use.

Sodium dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of *P. l. hutmanni* Venom and Venom Peak 2 (P2Plh)

Figure 2 shows the electrophoretic elution profiles of crude venom and P2Plh. Crude venom shows approximately 20 protein bands ranging from 7.6 to 206 kDa. P2Plh shows approximately 9 bands with molecular weights ranging from 7.6 to 117 kDa.

DEAE-Cellulose Chromatography Elution Profiles of *D. marsupialis* 0.15Dm Fraction

Figure 3 shows the chromatographic profiles of *D. marsupialis,* four peaks were obtained and tested by antiproteolytic activities.

SDS-PAGE from *D. marsupialis* Complete Sera and 0.15Dm Fraction

Figure 4 shows the electrophoretic elution profiles of *D. marsupialis* complete sera and 0.15Dm fraction. Complete sera shows protein bands ranging from 28 to >211 kDa. 0.15DM shows protein bands ranging from 40 to >211 kDa with a high abundance of protein in the 50–70 kDa range.

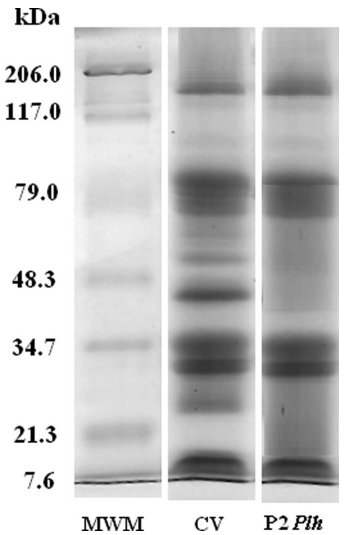

Figure 2: SDS-PAGE of crude *P. l. hutmanni* crude venom and P2Plh fraction from Sephadex G-100 molecular exclusion chromatography under non-reducing conditions. MWM: molecular weight markers (Bio-Rad, Hercules, CA, USA).

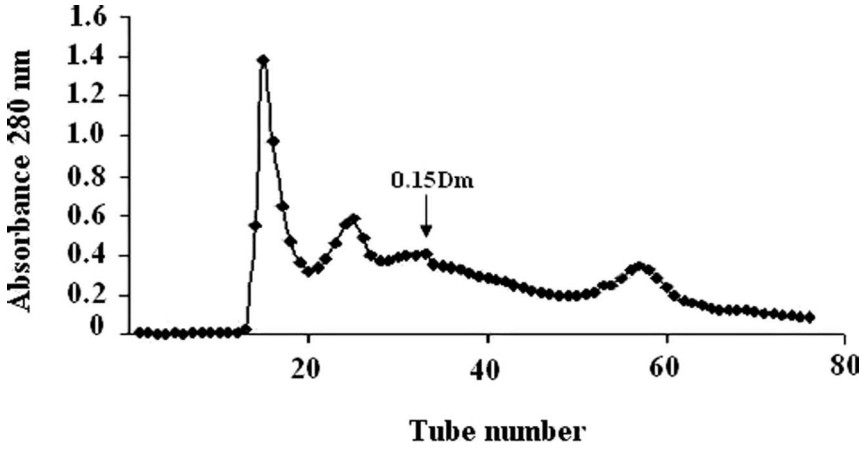

Figure 3: DEAE-Cellulose-PBS/NaCl chromatography elution profile of *D. marsupialis* sera.

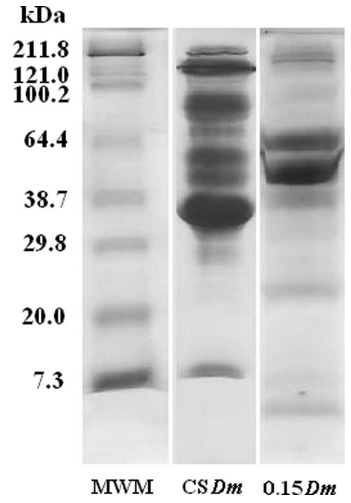

Figure 4: SDS-PAGE of *D. marsupialis* complete sera and 0.15 MDm fraction from DEAE-cellulose ion exchange chromatography using 12.5% gels under non-reducing conditions. MWM: molecular weight markers (Bio-Rad, Hercules, CA, USA).

Lethal Activity (LD_{50})

The calculated LD_{50} was 4.73 mg/Kg body weight.

Effective Dose (ED50)

The calculated ED_{50} of *D. marsupialis* sera for crude venom was 470 mg/kg weight. Alternatively, for P2Plh it was 198.0 mg/kg body weight.

Hemorrhagic Activity of *P. l. hutmanni* Venom and its Fraction P2Plh

The minimal hemorrhagic dose of *P. l. hutmanni* venom was 25 μg, while that for P2Plh was 15μg.

Neutralization of Hemorrhagic Activity of *P. l. hutmanni* Venom and its P2Plh Fraction by *D. marsupialis* serum and Fraction 0.15Dm

Only the immunoglobulin free serum fraction was able to protect against the hemorrhagic activity of crude venom and its fraction (P2Plh). The animals that received P2Plh fraction (15 μg) mixed with 0.15Dm (132 μg) did not present macroscopic lesions at the skin. The animals that received the P2Plh fraction alone or with saline solution had strong dermal hemorrhagic activity (Fig. 5).

Caseinolytic Activity of *P. l. hutmanni* Venom and its Fraction P2Plh

Porthidium lansbergii hutmanni P2Plh fraction degraded casein with an activity of 220 U/mg of protein, as compared to 200 U/mg with crude *P. l. hutmanni* venom and 207 U/mg with *Bothrops colombiensis* venom. The optimal pH for P2Plh caseinolytic activity was between pH 7 and 9. Crude venom and P2Plh fraction caseinolytic activity was a mean of two trials.

Neutralizing Effect on Caseinolytic Activity Induced by *P. l. hutmanni* Venom and P2Plh by 0.15Dm

The antiproteolytic activities of serum fractions from DEAE-cellulose ion exchange chromatography are shown in the Table 1.

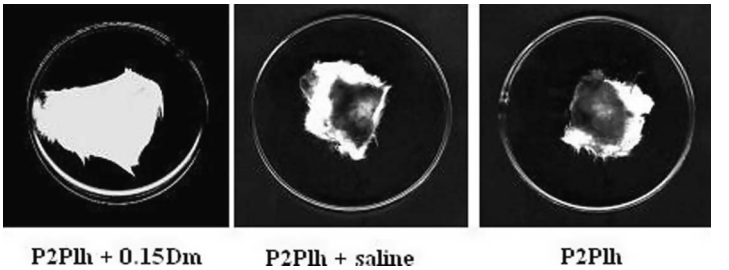

Figure 5: Neutralization of P2Plh venom-induced hemorrhagic activity by 0.15Dm.

Table 1: Neutralization of the hemorrhagic and caseinolytic activities of *P. l. hutmanni* crude venom and its P2Plh fraction by *D. marsupialis* sera and fractions collected from DEAE-cellulose ion exchange chromatography.

		***D. marsupialis* DEAE-cellulose fractions**			
Activity	**Crude sera**	**0.05 M**	**0.1 M**	**0.15 M[a]**	**0.2 M**
Antihemorrhagic activity against crude venom	+	–	–	+	–
Antihemorrhagic activity against P2Plh	+	–	–	+	–
Anticaseinolytic activity against crude venom	+	–	–	+	–
Anticaseinolytic activity against P2Plh	+	–	–	+	–

[a]Sera fraction (0.15Dm) inactivated 14.19 mg/Kg ($3LD_{50}$) of P2Plh venom fraction.
(+) Represents the presence of anti-hemorrhagic or anti-caseinolytic activities.
(–) Represents the absence of anti-hemorrhagic or anti-caseinolytic activities.

DISCUSSION

It has been proposed that venom metalloproteases are products with evolutionary variations frequently found in mammalian cells.[13] In addition, although not quite linked with the steps and evolutionary lines of the SVMPs, MMPs or ADAMs, the PLA_2s of venoms are also evolutionary variants of molecules, whose novel primordial function are merely digestive, and they have been useful in the progress and diversification of molecular strategies of many specialized cellular clusters.[1]

The functional similarities among the SVMPs, MMPs and their inhibitors may be the solution in the logical modulation of numerous biological activities associated with these molecules.[14] The biochemical origin for their action is the proteolytic damage of basal membrane and extracellular matrix adjacent capillaries and small vessels. These protease toxins could also hamper with coagulation, consequently supplementing loss of blood from the vasculature.[15] Equally important information is opportune when prognosing the fundamental growth of new therapeutic schemes established on the inhibition of venom's natural actions in any organism. Thus, the biological and biochemical consequences of snake venoms, implies that their accomplishment essentially depends on their proteolytic performance. This action has been mainly targeted for hemorrhagins[2,15] and myotoxins.[16]

The sera of *D. marsupialis*, a widespread opossum in South America, inhibit myonecrotic,[17] hemorrhagic,[18] lethal,[19] and hyperalgesic[20] effects observed in several Viperidae envenomations. While antihemorrhagic activity has been attributed to inhibitory factors present in *D. marsupialis* and

the sera of other mammals,[19, 21–30] quantitative disappointments, in the recuperation and purification of these resistance factors, from their natural supplies have also been encountered.

The genus *Didelphis* is a broadly distributed mammal in the American continent. The species of the genus distributed in Venezuela, are the *D. marsupialis* and *D. albiventris*, which are distributed in the Andes and the Coastal Ranges and the amazons' wild area. *Didelphis albiventris*, contrary to *D. marsupialis* is a perennial inhabitant of the whole Venezuelan geography.[31]

The first well-known report about the natural resistance of the family Didelphidae was carried out by Vellard.[32] He suggested that: "this immunity was acquired and constantly reinforced being able to transmit for inheritance." The studied *Didelphis* resisted the lethality and pathogen action of the intramuscular injection of venom, and their sera neutralized *Crotalus terrificus, Bothrops neuwiedii* and *B. jararaca* venoms.[32]

Werner and Vick[22] determined that the *D. virginiana* was resistant to *C. adamanteus, C. atrox, Agkistrodon c. contortrix, A. piscivorus*, *A. h. brevicaudata* and *A. bilineatus* venoms. In 1984, García and Pérez[26] purified and characterized an antihemorrhagic factor against *C. atrox* venom from the gray woodrat, *Neotoma micropus,* demonstrating it to have a pI of 4.1 and a molecular weight of 54 kDa.

Pifano et al.[25] reported the neutralizing property *D. marsupialis* sera against the *B. lanceolatus* hemorrhagic and proteolytic venom activity found in the DEAE-cellulose 0.1 M fraction, which was denominated F-0.1. Rodríguez-Acosta et al.,[18] running F-0.1 on SDS-PAGE and isolating all the bands by electroelution, achieved a 97 kDa band that neutralized *B. lanceolatus* venom hemorrhagic activity. Furthermore, Neves-Ferreira et al.[33] isolated DM40 and DM43 antihemorrhagic proteins by DEAE-Sephacel, Phenyl-Sepharose and Superdex 200 from *D. marsupialis* sera. Molecular masses of 44.8 and 47.3 kDa were obtained by SDS-PAGE, respectively for DM40 and DM43.

In the present work, we established the protective action of the factor (s) isolated from *D. marsupialis* sera on hemorrhagic and proteolytic effects induced by *P. l. hutmanni* venom and its fraction P2Plh. The 0.15Dm serum fraction ED_{50} for P2Plh (198 mg/kg) was 43.13% higher compared to that of crude sera (470 mg/kg), indicating that important inhibiting factors are concentrated in this sera fraction. The next step, currently being carried out by our laboratory, is the further purification and characterization of these inhibitors, which could improve the ED_{50}.

This antivenom fraction from opossum sera, when purified and characterized or a synthetic peptide, based on the aforementioned protein, could be of value in the medical management of *Porthidium* envenoming in humans and animals. Alternatively, since normal physiological process requires a precise balance

between metalloproteinases and tissue inhibitors of metalloproteinases, the comprehension of the biological interactions of both metalloproteinases found in snake venoms and metalloproteinases inhibitors found in these endothermic animals, could lead to a better understanding of the deregulation of the normal human or animal physiology caused by metalloproteinases.[30]

This study suggested that *P.l.hutmanni* venom activities could be inhibited by opossum sera fractions, and these molecules once isolated and charaterized may be available for the treatment of Lansberg's hognose pit viper snakebite accidents. Some sera fractions have anti-hemorrhagic effects and other fractions have anti-proteolytic effects on *P.l.hutmanni* venom, but the bioactive components are still not purified and further investigations and characterization of these sera molecules is necessary.

ACKNOWLEDGMENTS

Financial support was obtained from the Science and Technology Fund (FONACIT) programs (PG-2005000400; PG-2002000447) and CDCH de la Central University of Venezuela grants (PG-093453722003; PI-09-00-6712-2007), Caracas, Venezuela.

REFERENCES

1. Swenson, S.; Markland, F.S., Jr. Snake venom fibrin(ogen)olytic enzymes. Toxicon. **2005**; 45(8): 1021–1039.
2. Aguilar, I.; Guerrero, B.; Salazar, A.M.; Girón, M.E.; Pérez, J.C.; Sánchez, E.E.; Rodríguez-Acosta, A. Individual venom variability in the South American rattlesnake *Crotalus durissus cumanensis*. Toxicon. **2007**; 50(2): 214–224.
3. Salazar, A.M.; Rodríguez-Acosta, A.; Girón, M.E.; Aguilar, I.; Guerrero, B. A comparative analysis of the clotting and fibrinolytic activities of the snake venom (*Bothrops atrox*) from different geographical areas in Venezuela. Thromb. Res. **2007**; 120(1): 95–104.
4. Pifano, F.; Aguilar, I.; Girón, M.E.; Gamboa, N.; Rodríguez-Acosta, A. Natural resistance of opossum (*Didelphis marsupialis*) to the mapanare (*Bothrops lanceolatus*) snake venom. Roum. Arch. Microbiol. Immunol. **1993**; 52(2): 131–136.
5. Lizano, S.; Domont, G.; Perales, J. Natural phospholipase A_2 myotoxin inhibitor proteins from snakes, mammals and plants. Toxicon **2003**; 42(8): 963–977.
6. Vargas, A.M.; Finol, H.J.; Scannone, H.; Fernandez, I.; Rodriguez-Acosta, A. Effects of lansberg's hognose pit vipers (*Porthidium lansbergii hutmanni*) venom on renal ultrastructure in experimental mice. J. Submicrosc. Cytol. Pathol. In Press.
7. National Institutes of Health. *Principles of laboratory animal care*. Bethesda, MD: NIH; 1985.
8. Lowry, O.; Rosebrough, N.; Farr, L.; Randall R. Protein measurement with the folin phenol reagent. J. Bio. Chem. **1951**; 193(1): 265–275.
9. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **1970**; 227(5259): 680–685.

10. Spearman, C.; Karber, G. In: DJ Finney, Eds., *Alternative methods of analysis for quantal responses. Statistical methods in biological assays.* London: Charles Griffin and Co., Ltd.,; 1978; 1–78.

11. Omori-Satoh, T.; Sadahiro, S.; Ohsaka, A.; Murata, R. Purification and characterization of an antihemorrhagic factor in the serum of *Trimeresurus flavoviridis*, a crotalid. Biochim. Biophys. Acta. **1972**; 285(2): 414–426.

12. Lomonte, B.; Gutiérrez, J.M. Proteolytic activity of snake venoms of Costa Rica on casein. Rev. Biol. Trop .**1983**; 31(3): 37–40.

13. Jia, L.G.; Shimokawa, K.; Bjarnason, J.B.; Fox, J.W. Snake venom metalloproteinases: Structure, function and relationship to the ADAMs family of proteins. Toxicon. **1996**; 34(11–12): 1269–1276.

14. Zhang, D.; Botos, I.; Gomis-Rüth, F.X.; Doll, R.; Blood, C.; Njoroge, F.G.; Fox, J.W.; Bode, W.; Meyer, E.F. Structural interaction of natural and synthetic inhibitors with the venom metalloproteinase, atrolysin C (form d). Proc. Nat. Acad. Sci. U.S.A., **1994**; 91(18): 8447–8451.

15. Bjarnason, J.B.; Fox J.W. Hemorrhagic metalloproteinases from snake venoms. Pharmaco. Ther. **1994**; 62(3): 325–372.

16. Gutiérrez, J.M.; Lomonte, B. Phospholipase A2 myotoxins from *Bothrops* snake venoms. Toxicon. **1995**; 33(11): 1405–1424.

17. Melo, P.A.; Suarez-Kurtz, G. Release of sarcoplasmic enzymes from skeletal muscle by *Bothrops jararacussu* venom: Antagonism by heparin and by the serum of South American marsupials. Toxicon. **1988**; 26(1): 87–95.

18. Rodríguez-Acosta, A.; Aguilar, I.; Girón, M.E. Antivenom activity of opossum serum fraction. Toxicon. **1995**; 33(1): 95–98.

19. Neves-Ferreira, A.G.; Perales, J.; Ovadia, M.; Moussatché, H.; Domont, G.B. Inhibitory properties of the antibothropic complex from the South American opossum (*Didelphis marsupialis*) serum. Toxicon. **1997**; 35(6): 849–863.

20. Rocha, S.L.; Frutuoso, V.S.; Domont, G.B.; Martins, M.A.; Moussatché, H.; Perales, J. Inhibition of the hyperalgesic activity of *Bothrops jararaca* venom by an antibothropic fraction isolated from opossum (*Didelphis marsupialis*) serum. Toxicon. **2000**; 38(6): 875–880.

21. Werner, R.M.; Faith, R.E. Decrease in the lethal effect of snake venom by serum of the opossum, *Didelphis marsupialis.* Lab. Anim.Sci. **1978**; 28(6): 710–713.

22. Werner, R.M.; Vick, J.A. Resistance of the opossum (*Didelphis virginiana*) to envenomation by snakes of the family Crotalidae. Toxicon. **1977**; 15(1): 29–33.

23. Perez, J.C.; Pichyangkul, S.; Garcia, V.E. The resistance of three species of warmblooded animals to Western diamondback rattlesnake (*Crotalus atrox*) venom. Toxicon. **1979**; 17(6): 601–607.

24. Menchaca, J.M.; Perez, J.C. The purification and characterization of an antihemorrhagic factor in opossum (*Didelphis virginiana*) serum. Toxicon. **1981**; 19(5): 623–632.

25. Pifano, F.; Aguilar, I.; Giron, M.E.; Gamboa, N.; Rodriguez-Acosta, A. Natural resistance of opossum (*Didelphis marsupialis*) to the mapanare (*Bothrops lanceolatus*) snake venom. Roum. Arch. Microbiol. Immunol. **1993**; 52(2): 131–136.

26. Garcia, V.E.; Pérez, J.C. The purification and characterization of an antihemorrhagic factor in woodrat (*Neotoma micropus*) serum. Toxicon, **1984;** 22(1): 129–138.

27. Perales, J.; Amorim, C.Z.; Rocha, S.L.; Domont, G.B.; Moussatché, H. Neutralization of the oedematogenic activity of *Bothrops jararaca* venom on the mouse paw

by an antibothropic fraction isolated from opossum (*Didelphis marsupialis*) serum. Agents. Actions **1992**; 37(3–4): 250–259.

28. Rodríguez-Acosta, A.; Mondolfi, A.; Orihuela, R.; Aguilar, M. *¿Qué hacer ante un accidente ofídico?* Venediciones, Caracas, Venezuela, 1995.
29. Farah, M.F.; One, M.; Novello, J.C.; Toyama, M.H.; Perales, J.; Moussatché, H.; Domont, G.B.; Oliveira, B.; Marangoni, S. Isolation of protein factors from opossum (*Didelphis albiventris*) serum which protect against *Bothrops jararaca* venom. Toxicon. **1996;** 34(9): 1067–1071.
30. Pérez, J.C.; Sánchez, E.E. Natural protease inhibitors to hemorrhagins in snake venoms and their potential use in medicine. Toxicon. **1999**; 37(5): 703–728.
31. Linares, O.J. *Mamíferos de Venezuela*. Caracas: Sociedad Audubon de Venezuela/ British Petroleum, 1998.
32. Vellard, J. Resistance of Andine skunks to snake venom. Comp. Rend. Hebd. Séan. Académ.Sci. **1965;** 260(20): 5408–5410.
33. Neves-Ferreira, A.G.; Cardinale, N.; Rocha, S.L.; Perales, J.; Domont, G.B. Isolation and characterization of DM40 and DM43, two snake venom metalloproteinase inhibitors from *Didelphis marsupialis* serum. Biochim. Biophys. Acta. **2000**; 1474(3): 309–320.