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Neutralization of venoms from two Southern Pacific Rattlesnakes (*Crotalus helleri*) with commercial antivenoms and endothermic animal sera

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Abstract

The Southern Pacific Rattlesnake (*Crotalus helleri*) is found in southwestern California (USA), southward through north Baja California (MX) into the northern part of southern Baja California (MX). In this study, the venoms from two Southern Pacific Rattlesnakes were characterized. The two venoms were different in color, concentration, and enzyme activities. Two commercial antivenoms neutralized both *C. helleri* venoms differently. Antivipmyn (Fab_2H) and CroFab (FabO) neutralized both venoms but had different ED_{50}. Four times more Fab_2H antivenom was required to neutralize the *C. helleri* venom No. 011-084-009 than the venom from the snake No. 010-367-284. The hemorrhagic activity of two *C. helleri* venoms were neutralized differently by endothermic animal sera having a natural resistance to hemorrhagic activity of snake venoms. Opossums and Mexican ground squirrel sera did not neutralize the hemorrhagic activity of the venom No. 010-367-284. The sera of gray woodrats and hispid cotton rats neutralized all hemorrhagins in both *C. helleri* venoms. This is the first reported case in which opossum serum has not neutralized hemorrhagic activity of pit viper venom. Differences in the compositions of *C. helleri* venoms and their ability to be neutralized may help explain why snakebites are a difficult medical problem to treat and why effective polyvalent antivenoms are difficult to produce.

Keywords: *Crotalus helleri*; Antivenom; Venom; Natural resistance

1. Introduction

Chippaux and Goyffon (1991) reported the incidents of snakebites in the world to be 5,000,000 annually with approximately 40,000 deaths (Warrell, 1996). Physicians are generally at a disadvantage since they may lack information needed for proper treatment. In most cases, the species of the snake or the amount of venom injected by the snake is not known. Travel time to a medical facility may delay the administration of antivenom and tissue damage cannot be reversed. A physician has no assurance of the effectiveness of commercial antivenom. The composition of the particular venom causing the damage may be different from the venom used to produce the antivenom. All that can really be assumed is that polyvalent antivenom could have a high probability of neutralizing the venom and should reduce the recovery time and decrease the death rate.

Differences in venom toxicity and their ability to be neutralized by antivenom are important considerations in the manufacturing of an effective antivenom. When the exact species is not known or when it is not possible to differentiate between clinical symptoms, the best and most acceptable form of treatment is with polyvalent antivenom that neutralizes a wide variety of snake venoms (Theakston et al., 2002).

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The Southern Pacific Rattlesnake, *Crotalus helleri*, (see Douglas et al., 2002 for a phylogenetic revision of this species) has myotoxic, neurotoxic, and hemorrhagic components in its venom (Metsch et al., 1984; Schaeffer et al., 1999). This study reports considerable variation in venoms from two different *C. helleri* snakes and in their ability to be neutralized by two commercial antivenoms and sera from five different endothermic animals. Understanding differences in intraspecific venom variation may give insights to the development and testing of more effective antivenoms.

2. Methods

2.1. Venom extraction

Venoms were extracted from two Southern Pacific Rattlesnakes, *C. helleri*, taken from the same location in southern California. Both snakes are housed in the Natural Toxins Research Center (NTRC) at Texas A&M University-Kingsville. The venom samples were centrifuged at 500*g* for 10 min, and filtered using a Millipore MillexHV 0.45 μm filter unit. The venoms were lyophilized and stored at −90 °C. The *C. helleri* producing yellow venom has a pit tag No. 010-367-284, and the *C. helleri* producing white venom has a pit tag No. 011-084-009. Information about these snakes can be found on the Internet using the pit tag numbers (http://ntrc.tamuk.edu).

2.2. Determination of venom and sera concentrations

The venom concentration was determined by lyophilizing venom and weighing the dry mass. The ratio of dry venom (mg) per volume (ml) was considered the concentration for crude venom. The protein concentrations of venom fractions, antivenom and animal sera were determined by the standard method of 280 nm (Layne, 1957).

2.3. Antivenoms

The neutralization of yellow and white venoms was compared with two commercial antivenoms. Antivipmyn (Fab_2H) is an equine origin antivenom produced by Instituto Bioclon in Mexico (lot No. B-2E-05, exp. date: May, 2006). The snake venoms used to produce the Fab_2H were *Crotalus durissus durissus* and *Bothrops asper* and were $(Fab')_2$ fragments. The second antivenom used was Crotalidae Polyvalent Immune Fab Ovine (FabO) and produced by Therapeutic Antibodies, Inc., London, England (lot No. CRO/140/008/001, exp. date: not available). Therapeutic Antibodies is now called Protherics, Inc., and is located in Brentwood, TN. The snake venoms used to produce FabO were *Crotalus atrox* (Western Diamondback Rattlesnake), *Crotalus adamanteus* (Eastern Diamondback Rattlesnake), *Crotalus scutulatus scutulatus* (Mojave Rattlesnake), and *Agkistrodon piscivorus piscivorus* (Eastern Cottonmouth). FabO is an affinity purified Fab fragment.

2.4. Sera

Five different animal sera known to have metalloproteinase inhibitors were used to neutralize hemorrhagic activity of both *C. helleri* venoms (Perez et al., 1978; Rodriguez-Acosta et al., 1995; Pérez and Sánchez, 1999). *Didelphis virginiana* (Virginia opossum), *Neotoma micropus* (gray woodrat), *Sigmodon hispidus* (hispid cottonrat), and *Spermophilus mexicanus* (Mexican ground squirrel) blood was collected *via* heart puncture from animals. These four animals were captured in Kleberg County, TX. Serum from *Didelphis marsupialis* (South American opossum) was obtained by the Instituto de Medicina Tropical, Universidad Central de Venezuela in Caracas. The blood was allowed to coagulate for 24 h at 4 °C. The blood was then centrifuged at 10,509*g* for 10 min using a Beckman Avanti™ 30 Centrifuge at 4 °C and filtered through a 0.45 μm Millex HV filter.

2.5. Electrophoretic titration

Electrophoretic titration (ET) curves were established using a Pharmacia PhastSystem for both *C. helleri* venoms and were used to determine the optimal conditions for separation of venom by ion exchange chromatography. The venom samples were dissolved in Milli-Q water. A pH gradient of 3–9 was established on a polyacrylamide IEF 3-9 PhastGel. The gels were rotated 90° and 17.5 μg of both *C. helleri* venom were applied to separate gels. The gels were stained with silver nitrate.

2.6. Anion exchange chromatography

Eight milligrams of crude yellow and white venoms were injected into a Waters Protein-Pak DEAE 5PW (7.5×75 mm^2) HPLC column. Fractions were eluted using a 0.02 M Tris–HCl buffer, pH 8.0, and 0.5 M NaCl gradient over a 1 h period with a flow rate of 1 ml/min. A Waters 484 tunable detector was used to monitor absorbancies at 280 nm. Millennium software v.4.0 was used to control the pumps and store the data. Fractions were stored at −90 °C. Protein concentrations were determined by the standard method of 280 nm. Two separations were done for both venoms.

2.7. Hemorrhagic assay

Hemorrhagic activity was determined by a modification of Omori-Satoh et al. (1972). A series of dilutions were made for each venom sample and 0.1 ml of each dilution was injected intra-cutaneously (i.c.) into the back of a depilated New Zealand (*Orytolagus cuniculus*) rabbit to determine hemorrhagic activity. The rabbit was sacrificed after 24 h, the skin removed and the diameter of

hemorrhagic spots measured. The minimal hemorrhagic dose (MHD) was defined as the amount of venom protein causing a 10 mm hemorrhagic spot. This assay was repeated three times.

2.8. Antihemorrhagic assay

Equal amounts of a 2MHD solution of crude venoms were mixed with equal volumes of the two antivenoms and incubated for 1 h at 25 °C. The same procedure was used to test five crude animal sera for antihemorrhagic activity. The back of a New Zealand rabbit (*O. cuniculus*) was depilated and 0.1 ml of each dilution was injected i.c., thus injecting only one MHD. The rabbit was sacrificed after 24 h and the hemorrhagic spots were measured. The antihemorrhagic dose (AHD) is defined as the amount (μg) of antivenom inhibiting 50% of 1MHD of snake venoms. The lower the dose, the more efficient the antivenom. This assay was repeated three times.

2.9. Fibrinolytic assay

A method modified from Bajwa et al. (1980) was used to measure fibrinolytic activity of the fractions obtained from the venoms. Fibrinogen (9.4 mg/ml, 300 μl) and thrombin solutions (38.5 U/ml, 10 μl) were added to each well of a 24-well plate. The plate was gently shaken to mix the two solutions. The solution in the plate was allowed to solidify at room temperature. The plate was then incubated for 3 h at 37 °C. Ten microliters of each venom fraction, various concentrations of crude yellow and white venoms were added individually in the center of each well and incubated for 15 h at 37 °C. Seven hundred microliters of 10% trichloroacetic acid (TCA) were placed in each well and then decanted after 10 min. The minimal fibrinolytic dose (MFD) was determined as the amount of venom (μg) that produced a clear zone with a 5 mm diameter on the fibrin clot. This assay was repeated three times.

2.10. Gelatinase assay

A method modified from Huang and Perez (1980) was used to test gelatinase activity of crude venoms and venom fractions. Kodak X-OMAT scientific imagining film was washed with Milli-Q water and incubated at 37 °C for 30 min. After incubation, the film was dried and 20 μl of samples were placed on the film. Hydrolysis of gelatin on the film was determined by washing the film with tap water after 4 h incubation at 37 °C in a moist incubator. The clearing of the gelatin on the film indicated a positive result. The minimal amount of venom (μg) that caused a clear zone with a 5 mm diameter on the film was defined as the minimal gelatinase dose (MGD). This assay was repeated three times.

2.11. Hide powder azure assay

A modified method of Rinderknecht et al. (1968) was used to test proteolytic activity of venom fractions. The hide powder azure was ground with a mortar and pestle to obtain uniform particles. For every venom sample and venom fractions, 8 mg of hide powder azure were mixed with 2 ml of 0.02 M Tris–HCl buffer, pH 8.0, and 100 μl of various concentrations of crude venom or venom fractions were added to the vial. Each vial was incubated for 1 h at 37 °C and agitated at 5 min intervals. After incubation, each vial was centrifuged at 420*g* for 5 min. The supernatant was transferred into a different vial and the absorbaency measured at 595 nm. An absorbancy reading higher than 0.1 was considered positive. The minimal amount of crude venom (μg) required to give an absorbance reading of 0.1 is defined as the minimal hide powder dose (MHPD). This assay was repeated three times.

2.12. Hydrolysis of B-chain of insulin

A method described by Sánchez et al. (2001) was used to determine proteolytic activity on B-chain of insulin. A Beckman Capillary Electrophoresis P/ACE 5500 was used to detect the proteolytic activity of both venoms and their anion exchange fractions. Ten microliters of sample (0.03 mg/ml) were incubated for 10 min at 25 °C with 10 μl (0.5 mg/ml) of B-chain of insulin and 10 μl of 100 mM Borate buffer, pH 8.3. The mixture was immediately separated at 20 kV, 19.5 μA for 10 min using a 100 mM Borate buffer, pH 8.3 through a 75 μm ID × 50 cm (100 × 800 aperture) free zone capillary. A P/ACE UV absorbance detector at 214 nm was used to detect the peptides. Results were recorded by the percent reduction in absorbance of B-chain insulin by the following equation: $[(C - E/C)] \times 100$; where C is the absorbance units of B-chain control; E is the absorbance units of B-chain incubated with venom or venom fractions.

2.13. Fibrinogenolytic

A modified method of Ouyang and Teng (1976) was used to test the fibrinogenolytic activity of crude venoms and their individual anion exchange fractions. Two-hundred microliters of human fibrinogen solution (10 mg/ml) in 20 mM Tris/HCl buffer, pH 8.0, and 100 μl of *C. helleri* crude venom or venom fractions (0.03 mg/ml) were mixed and incubated at 37 °C. Fractionated venom samples were incubated for 4 h. Twenty microliter aliquots were taken after seven different incubation periods (initial, 2, 10, 30, 60, 120 and 240 min) and added to 20 μl of 20 mM Tris/HCl buffer, pH 8.0 containing 2 mM EDTA, 5% (w/v) SDS, 0.02% (w/v) bromophenol blue and 10% (v/v) beta-mercaptoethanol. The samples were then boiled for 3 min in a water bath and crude venom and fractions were analyzed by SDS using a homogenous PhastGel 12.5 on

a Pharmacia PhastSystem. The gels were stained with silver nitrate. The positive results were recorded by visible reduction of the α chain of fibrinogen on the gel.

2.14. Lethal dose assay (LD_{50})

Crude venoms were dissolved in physiological saline at the highest concentrations of venom that were used for injection (six different concentrations using two-fold serial dilutions were prepared). All solutions during the experiment were kept at 0 °C and warmed to 37 °C just before being injected into mice. For each concentration, eight 18–20 g, female mice were injected 0.2 ml into their tail veins. The injections were administered using a 1-ml syringe fitted with a 30-gauge, 0.5 in. needle. Saline controls were used. The endpoint of lethality of the mice was determined after 48 h. The LD_{50} was calculated by a method developed by the NTRC and found on the Internet (http://ntri.tamuk.edu/cgi-bin/ld50/ld50). This LD_{50} calculator is based on calculations used by Reed and Muench (1938).

2.15. Serum protection test (ED_{50})

For each antivenom concentration, eight mice were challenged with a mixture of $3 \times LD_{50}$ of venom. Antivipmyn (Fab_2H) and Crofab (FabO) antivenoms were used. The ED_{50} for both Fab_2H and FabO were determined for both venoms. Six doses of antivenom were used. Stock venom solutions containing $30 \times LD_{50}$ were prepared at 0 °C before being used. For each group of mice, equal volumes of venom and antivenom were mixed and incubated at 37 °C for 30 min. Each mouse was injected with 0.2 ml of venom/antivenom mixture into the tail vein. The mice were observed for 48 h and the percent survival and ED_{50} were calculated. Saline controls and antivenom controls were used. The ED_{50} was calculated by a method developed by the NTRC and is found on the Internet (http://ntri.tamuk.edu/cgi-bin/ld50/ed50). This ED_{50} calculator is based on calculations used by Reed and Muench (1938).

2.16. Inhibition of platelet aggregation

Platelet aggregation was performed with a Chrono-Log Whole Blood Lumi Aggregometer [Ca^{2+}] by impedance. Four hundred microliters of saline and 450 μl of whole citrated human blood solution were added to a cuvette and incubated at least 5 min. Five microliters of anion exchange venom fractions collected from both venoms were added to the cuvette and incubated for 2 min. Electrodes measuring impedance were inserted in the cuvette. After 1.5 min, 20 μl of a 1 mM ADP solution was added to initiate platelet aggregation. A 0.02 M Tris–HCl buffer, pH 8.0 was used as a control.

3. Results

The two *C. helleri* venoms used in this study were different in their color, protein concentration, HPLC and ET profiles and enzymatic activities (Table 1). For the sake of simplicity, the venoms will be referred to as yellow and white. Crude yellow venom from *C. helleri* had a concentration four times higher (540 mg/ml) than white venom (135 mg/ml). The yellow venom was more hemorrhagic having a MHD of 0.71 μg, while the white venom was 2.06 μg. The white venom had higher gelatinase activity with a MGD of 47.5 μg, while the yellow venom was 83 μg. The white venom was more fibrinolytic with a MFD of 25 and 100 μg for the yellow venom. The yellow venom had lower minimal hide powder (MHPD) activity (400 μg) while the white venom had a higher MHPD (133 μg). White venom had a higher proteolytic activity when B-chain insulin was used as the substrate. Fifty-six percent of the B-chain of insulin was digested with the white venom and 38% with the yellow venom. The yellow venom

Table 1
Comparison of two samples of *C. helleri* venoms

Venom color	Concentration (mg/ml)	MHD[a] (μg)	MGD[b] (μg)	MFD[c] (μg)	MHPD[d] (μg)	B-chain[e] (%)	LD_{50}[f]	Fibrinogenolytic (min)[g]
Yellow	540	0.71 ± 0.14	83.0 ± 28	100 ± 0	400 ± 0	38 ± 7.70	1.84	120
White	135	2.06 ± 0.89	47.5 ± 4.3	25.0 ± 0	133 ± 0	56 ± 14.3	2.95	240

[a] The minimal amount of venom protein injected into the back of depilated rabbit causing a 10 mm hemorrhagic spot in diameter. Standard deviations were used to measure statistical differences in all activities.
[b] The minimal amount of venom protein (μg) that causes a digestion of gelatin on imaging film.
[c] The minimal amount of venom protein (μg) that causes a 5 mm clearing of fibrin plate.
[d] The minimal amount of venom protein (μg) that gives an absorbance reading (595 nm) of 0.1.
[e] Percentage of cleavage of B-chain insulin with a venom concentration of 0.03 mg/ml. The peak absorbances were used to measure activity.
[f] The LD_{50} is the concentration of venom (mg/kg body weight) required to kill 50% of the BALB/c mice.
[g] The time required to cleave α chain of fibrinogen with a venom concentration of 0.03 mg/ml.

Table 2
Comparison of the neutralization of hemorrhagic activity in *C. helleri* venoms with two different antivenoms, Antivipymn (Fab_2H) and CroFab (FabO)

Venom color	AHD[a] Fab_2H (μg)	AHD[a] FabO (μg)
Yellow	11.0 ± 3.8	26.5 ± 0
White	14.2 ± 10.7	21.7 ± 7.39

[a] The amount of antivenom (μg) that neutralizes 50% of 1MHD of venom. The AHD is calculated by dividing the starting concentration of antivenom by the antihemorrhagic titer that neutralizes 50% of 1MHD and then multiplying by the amount of volume injected into the back of a depilated rabbit. Standard deviations were used to measure statistical differences in all activities.

was more toxic with a LD_{50} 1.84 mg/kg body weight and LD_{50} of 2.95 for the white venom. The yellow venom cleaved the α chain in fibrinogen in 120 min and white venom required 240 min to reduce the same amount of α chain. The yellow venom was more toxic and hemorrhagic (Table 1), while the white venom was more effective in cleaving gelatin, fibrin and B-chain of insulin.

The hemorrhagic activities of yellow and white venoms were not neutralized equally with Antivipymn (Fab_2H) and CroFab (FabO) antivenoms. Antivipymn was more effective in neutralizing hemorrhagic activities of both yellow and white venoms (Table 2). Four of the five animal sera neutralized hemorrhagic activity of the white venom, with *S. mexicanus* serum having the highest antihemorrhagic activity; however, *D. virginiana*, *D. marsupilis* and *S. mexicanus* sera did not neutralize hemorrhagic activity of yellow venom (Table 3).

Fab_2H neutralized the lethal activity of the yellow venom more effectively than FabO, but FabO neutralized the white venom better than antivipymn (Table 4). The ED_{50} for Fab_2H was 83.6 mg/kg body weight using yellow venom, while white venom required a higher ED_{50} of 357.3. The ED_{50} for FabO against yellow venom was 139.5 mg/kg body weight, while the ED_{50} against white venom was 108.4 (Table 4).

The ET profiles and HPLC profiles were different for both venoms (Figs. 1a and 2a). The ET curves for the yellow venom revealed multiple bands with pI ranging from 3 to 8 (Fig. 1b). The ET profile for white venom was less complex and had pI that ranged from 3.5 to 6 (Fig. 2b). The yellow venom had a more complex HPLC profile than the white venom when fractionated with a DEAE column (Figs. 1a and 2a). None of the fractions for yellow venom contained fibrinolytic or hide powder activities. Crude yellow *C. helleri* venom had only moderate fibrinolytic and hide powder activities (Table 1). The HPLC fractions for white venom were also devoid of fibrinolytic activity; however, fraction 1 contained hide powder and gelatinase activities (Fig. 2a). Crude, white venom had weak fibrinolytic activity (Table 1). Gelatinase activity was also present in one of the fractions of the yellow venom, but unlike the white venom, the fraction containing gelatinase activity was highly acidic. All acidic peaks including fraction 11 of the white venom were hemorrhagic (Fig. 2a), while peaks 4 and 5 did not contain hemorrhagic activity (Fig. 1a). Furthermore, all acidic peaks from the white venom hydrolyzed B-chain of insulin (Fig. 2a), and only peaks 6–10 and 12 of the yellow venom hydrolyzed B-chain of insulin (Fig. 1a). Fractions 1, 4, 5, 8 and 11 of the yellow venom inhibit platelet aggregation, while none of the fractions of the white venom affected normal platelet aggregation (Figs. 1 and 2).

4. Discussion

Johnson et al., 1987 reported that *C. helleri* venoms were different in each gland. This interesting finding provokes some questions about in vivo synthesis of snake venom. Is variation in venoms due to (1) genetic differences in snakes, (2) gene regulation, (3) a dysfunctional gland caused by injury or disease, (4) autolysis giving rise to different forms of the same venom protein, or (5) a combination of all or several of these factors? Since the color and the viscosity of the venoms were different, the purpose of this study was to determine if there were other differences in the venoms and if they were neutralized differently by commercial antivenom and animal sera.

Geographical variations in snake venoms (Glenn and Straight, 1977, 1978, 1989; Adame et al., 1990) as well as

Table 3
Comparison of the neutralization of hemorrhagic activity in *C. helleri* venoms with sera of five different endothermic animals

Venom color	AHD[a] Dv (μg)	AHD[a] Dm (μg)	AHD[a] Nm (μg)	AHD[a] Sh (μg)	AHD[a] Sm (μg)
Yellow	N/A	N/A	62.3 ± 76.7	18.1 ± 0	N/A
White	227 ± 131	N/A	208 ± 164	290 ± 0	146 ± 131

The AHD is calculated by dividing the starting concentration of antivenom by the antihemorrhagic titer that neutralizes 50% of 1MHD and then multiplying by the amount of volume injected into the back of a depilated rabbit. Standard deviations were used to measure statistical differences in all activities. N/A, no activity. *D. virginiana* (Dv), *D. marsupialis* (Dm), *N. micropus* (Nm), *Sigmidon hispdus* (Sh) and *S. mexicanus* (Sm).

[a] The amount of animal sera (μg) that neutralizes 50% of 1MHD of venom.

Table 4
Comparison of LD_{50} and ED_{50} of *C. helleri* venoms with two different antivenoms, Antivipymn (Fab_2H) and CroFab (FabO)

Venom color	LD_{50}[a]	ED_{50}[b] Fab_2H	ED_{50}[b] FabO
Yellow	1.84	83.6	139.5
White	2.95	357.3	108.4

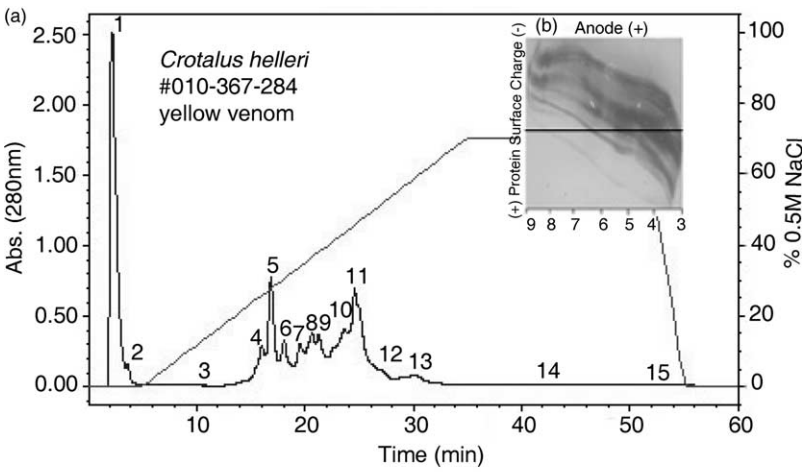
[a] The LD_{50} is the concentration of venom (mg/kg body weight) required to kill 50% of the BALB/C mice injected i.v. with 0.2 ml of the various snake venoms. LD_{50} was calculated using the LD_{50} calculator on the Natural Toxins Research Center's homepage at http://ntri.tamuk.edu/cgi-bin/ld50/ld50.

[b] The $ED_{50}$is expressed as milligram of antivenom/kg of mouse body weight; ED_{50} values were determined against $3 \times LD_{50}$ of venoms.

variations due to environmental factors are documented in the literature (Moura-da-Silva et al., 2003). Due to these variations, commercial antivenoms may not be effective in neutralizing specific venoms (Sánchez et al., 2003). Therefore, it is important to collect snake venoms throughout the geographical range, including all ages and both genders for the production and testing of polyvalent antivenom.

The *C. helleri* venoms used in this study were considerably different with respect to color, concentration, toxicity, HPLC and ET profiles and enzymatic activities. The DEAE/HPLC profiles had a large basic peak in its void volume (21% of the total protein) that was not significantly represented in the ET profile (Fig. 1a). Refractionation of the void volume under the same conditions revealed that acidic proteins were found in the void volume (refractionation data not shown). These results indicate that 8 mg of protein was enough to saturate the DEAE column. The maximum column capacity of an ion exchange column is based on protein size and smaller amounts of low molecular mass proteins will saturate a DEAE column faster.

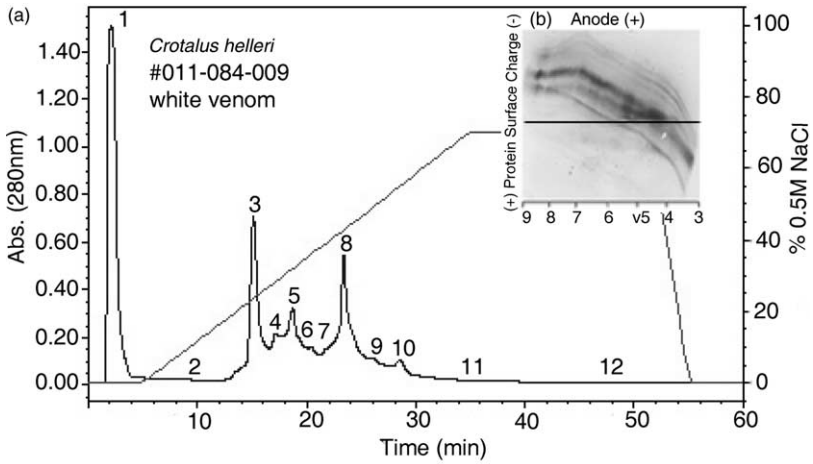
The crude yellow venom was four times more concentrated than the white venom. In addition, white and yellow *C. helleri* venoms were neutralized with antivenom



(c)

Assays	Fraction # 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Fibrinolytic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Hemorrhagic	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-
Hide powder	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of B-chain of Insulin	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-
Fibrinogenolytic	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-
Inhibition of Platelet Aggregation	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-

Fig. 1. Anion exchange chromatography and electrophoretic titration profile of *C. helleri* yellow venom. (a) A total of 8 mg of venom was injected into a Waters PROTEIN-PAK DEAE 5PW (7.5×75 mm^2) HPLC column. The fractions were eluted using a 0.02 M Tris–HCl buffer, pH 8.0, and a 0–0.5 M NaCl salt gradient was used. The collection required 60 min with a flow rate of 1 ml/min. A Waters 484 tunable detector was used to monitor absorbancies at 280 nm. Millennium software v.4 was used to control the pumps and store the data. Fractions of various volumes were collected and tested for activity. (b) Electrophoretic titration profile determined the conditions for ion exchange chromatography. A pH gradient of 3–9 was established on a Pharmacia PhastGel System and 17.5 μg of crude venom was applied to the gel. The gel was stained with silver nitrate. (c) Table of HPLC fractions and their activities tested. A '+' represents detectable activity whereas a '−' indicates the absence of detectable activity.



(c)

Assays	Fraction # 1	2	3	4	5	6	7	8	9	10	11	12
Fibrinolytic	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	+	-	-	-	-	-	-	-	-	-	-	-
Hemorrhagic	-	-	+	+	+	+	+	+	+	+	+	-
Hide powder	+	-	-	-	-	-	-	-	-	-	-	-
Hydolysis of B-chain of Insulin	-	-	+	+	+	+	+	+	+	+	-	-
Fibrinogenolytic	-	-	-	+	-	+	+	-	-	+	-	-
Inhibition of Platelet Aggregation	-	-	-	-	-	-	-	-	-	-	-	-

Fig. 2. Anion exchange chromatography and electrophoretic titration profile of *C. helleri* white venom. (a) A total of 8 mg of venom was injected into a Waters PROTEIN-PAK DEAE 5PW (7.5 × 75 mm^2) HPLC column. The fractions were eluted using a 0.02 M Tris–HCl buffer, pH 8.0, and a 0–0.5 M NaCl salt gradient was used. The collection required 60 min with a flow rate of 1 ml/min. A Waters 484 tunable detector was used to monitor absorbancies at 280 nm. Millennium software v.4 was used to control the pumps and store the data. (b) Electrophoretic titration profile determined the conditions for ion exchange chromatography. A pH gradient of 3–9 was established on a Pharmacia PhastGel System and 17.5 μg of venom was applied to the gel. The gel was stained with silver nitrate. (c) Table of HPLC fractions and their activities tested. A ‘ + ’ represents detectable activity whereas a ‘ − ’ indicates the absence of detectable activity.

and animal sera differently. The white *C. helleri* venom was 1.6 times less toxic than the yellow venom as determined by the LD_{50} (Table 4). In a study done by Consroe et al. (1992), the LD_{50} for the *C. helleri* venom was 3.48 mg/kg body weight, which is not a major difference from the results of this study. However, there was a substantial difference between the ED_{50} using FabO antivenom against the *C. helleri* venoms used in this study and the ED_{50} for *C. helleri* venom in the Consroe et al. (1995). In the Consroe study, FabO antivenom was not able to neutralize the LD_{50} for *C. helleri* venom very effectively. The ED_{50} in the Consroe study was 849.8 mg/kg body weight, while in this study, the ED_{50} for FabO against yellow and white venoms were 139.5 and 108.4 mg/kg body weight, respectively. The FabO antivenom was 12 times more effective in neutralizing the LD_{50} of *C. helleri* venoms used in this study than the venom used in the Consroe study. The two studies used similar protocols but a different strain of mice and different venoms were used. Furthermore, Consroe et al. (1995) used pooled venom and in this study two different *C. helleri* venoms were used that were different in color concentration and proteolytic activities. Sánchez et al. (2003) reported that the ED_{50} for FabO against pooled *C. helleri* venom was 70 mg/kg body weight which are closer to the results of this study. Fab_2H neutralized the LD_{50} better than FabO for the more toxic yellow venom (Table 4) while FabO was three times more effective than Fab_2H in neutralizing the LD_{50} of the white venom.

Furthermore, the hemorrhagic activities of both venoms were neutralized differently with sera of animals having a natural resistance against snake venoms. The natural resistance of certain mammals to snake vemoms was first documented by Phisalix and Bertrand (1895). More recently, others have shown that certain endothermic animals have the ability to neutralize hemorrhagic activity in snake venoms (Kilmon, 1976; Ovadia and Kochva, 1977; Werner and Vick, 1977; De Wit and Westrom, 1985; Perales et al., 1986; Rodriguez-Acosta et al., 1995). Perez et al. (1978) reported 14 of 40 species of endothermic animals tested, neutralized hemorrhagic activity of Western Diamondback Rattlesnake (*C. atrox*) venom. Soto et al. (1988) analyzed 35 different snake venoms for proteolytic

and hemorrhagic activity and 85% of the snake venoms tested were hemorrhagic. All hemorrhagic venoms were neutralized by crude woodrat (*N. micropus*) and opossum (*D. virginiana*) sera. However, in this study, the hemorrhagic activity of the yellow venom was not neutralized by either opossums (*D. virginiana* and *D. marsupialis*) sera (Table 3). This has been the first study to report that the Virginia opossum serum was unable to neutralize a hemorrhagic venom of *C. helleri*. In addition, the hemorrhagic activity of yellow venom was not neutralized by the Mexican ground squirrel (*S. mexicanus*) serum. *D. marsupialis* serum was the only serum not capable of neutralizing the hemorrhagic activity of the white venom. The white and yellow *C. helleri* venoms were neutralized differently with all animal sera used in this study (Table 3).

The conclusions support the theory that there are differences found in venom within the same species and the venoms are not neutralized equally. Understanding geographical and environmental variations in snake venoms may be crucial in developing better therapeutic agents for treating snakebites. The results of this study may help explain why treatment of snakebites is a difficult medical problem and why certain antivenoms may have some difficulties in treating all bites from snakes for which it was produced.

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