Lymphology 45 (2012) 144-153

LYMPHATIC ROUTE OF TRANSPORT AND PHARMACOKINETICS OF *MICRURUS FULVIUS* (CORAL SNAKE) VENOM IN SHEEP

D. Paniagua, L. Jiménez, C. Romero, I. Vergara, A. Calderón, M. Benard, M.J. Bernas, H. Rilo, A. de Roodt, G. D'Suze, M.H. Witte, L. Boyer, A. Alagón

Instituto de Biotecnología (DP,LJ,IVAC,MB,AA), Universidad Nacional Autónoma de México, Cuernavaca, México; Departamento de Producción Agrícola y Animal (CR), Universidad Autónoma Metropolitana, Unidad Xochimilco, México, DF; Department of Surgery (MJB,HR,MHW), University of Arizona, Tucson, AZ, USA; Instituto Venezolano de Investigaciones Científicas (IVIC) (GDS), Caracas, Venezuela; Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina (AdeR); Venom Immunochemistry, Pharmacology, and Emergency Response (VIPER) Institute (LB), University of Arizona, Tucson, AZ, USA

ABSTRACT

The contribution of the lymphatic system to the absorption and systemic bioavailability of Micrurus fulvius *venom after subcutaneous (SC) administration was assessed using a central lymph-cannulated sheep model.* Micrurus fulvius *venom was administered either by intravenous bolus (IV) or subcutaneous injection (SC) in 12 sheep with and without thoracic duct cannulation and drainage. Venom concentration in serum and lymph was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) in samples collected over a 6-hour period and in tissues harvested at the end of the experiment. Pharmacokinetic parameters were determined by a non-compartmental analysis. In the lymphatic cannulated group, over the 6 hours after the venom was administered, 69% of administered dose was accounted for in blood (45%) and lymph (25%). Negligible levels of venom were detected in organs and urine implying that the steady state observed after SC administration is maintained by a slow absorption process. Comparison of kinetics of the thoracic duct cannulated and non-cannulated groups showed that lymphatic absorption contributed in an important way to maintenance of this steady state. These results show that the limiting process in the pharmacokinetics of* Micrurus fulvius *venom following SC administration is absorption, and that the lymphatic system plays a key role in this process.*

Keywords: *Micrurus fulvius*, coral snake, venom, lymphatic absorption, pharmacokinetics, thoracic duct, sheep

Micrurus fulvius is a coral snake endemic to the Southeastern United States. Bites are rare, but they cause local tissue reaction or pain at the bite site followed by a progressive paralysis that can evolve to respiratory arrest and death (1). Symptoms may be delayed for 18 hours after the bite (2) making diagnosis of severe envenomation challenging.

The venom is a complex mixture of low and high molecular weight proteins with different physicochemical properties and biological activities. Biologically active components include hyaluronidase (3), nucleotidase, phosphodiesterase and aminopeptidase (4). The primary toxins responsible for envenomation symptoms

include two groups of neurotoxins (5-10). The first group, α-neurotoxins, are small proteins approximately 7 kDa in size which block the nicotinic acetylcholine receptor (11,12). The second, ß-neurotoxins, are phospholipases A2 of approximately 13 kDa and these inhibit presynaptic acetylcholine release (13,14).

Coral snakes have short fixed fangs that require a chewing action to inject the venom (2). This feature suggests that injection of venom is by the SC route. Absorption kinetics of subcutaneously injected proteins have been studied extensively since 1963 (15), and more recently (16-20). Depending largely on size following SC administration, the bioavailability of these proteins varies from 20% to 100%, with a relatively slow rate of absorption evidenced by a prolonged terminal half-life in comparison to that observed after IV administration. Maximum plasma concentrations occur from 2 to 20 hrs after SC dosing (21). These observations are directly related to the way that proteins are absorbed from the injection site to the systemic circulation. When proteins are deposited in the interstitial space, they diffuse until reaching a vessel with permeable endothelium where they can be absorbed. The permeability of the blood vascular endothelium is restricted by size of intercellular junctions which allow exchange of gas and small molecules. Transport in the blood capillaries is limited by size and as solute size increases, permeability decreases (22). In contrast, the initial capillaries of the lymphatic system can have large gaps between adjacent endothelial cells allowing the entrance of larger molecules and even cells (23). Supersaxo and colleagues reported a linear relationship between molecular weight and lymphatic absorption for molecules of 0.2 to 19.0 kD (24).

While some earlier work has been reported on venom absorption kinetics in animals (25, 26), and large animals including sheep lymphatics have been cannulated in a wide variety of studies (17- 21,24, 27-29), the convergence of these approaches to describe absorption of venom via the lymphatics route has not been explored, although the venom absorption via lymphatic system has been suggested since 1939 (30). Since lymph has a slow flow and small volume, absorption by this route should affect residence time in the body as well as absorption rate. Because much is unknown about the reservoir depots and transport of venom in the clinical setting and these parameters are largely responsible for the clinical course, a large animal model is needed to explore these parameters. Our model of envenomation in sheep offers the ability to study these depot and transport parameters as well as to define more closely the pharmacokinetics. Therefore, the aim of this study was to establish the pharmacokinetics of coral snake venom when injected IV and SC, and to assess the contribution of the lymphatic system to the absorption and systemic bioavailability of the *Micrurus fulvius* venom after SC administration.

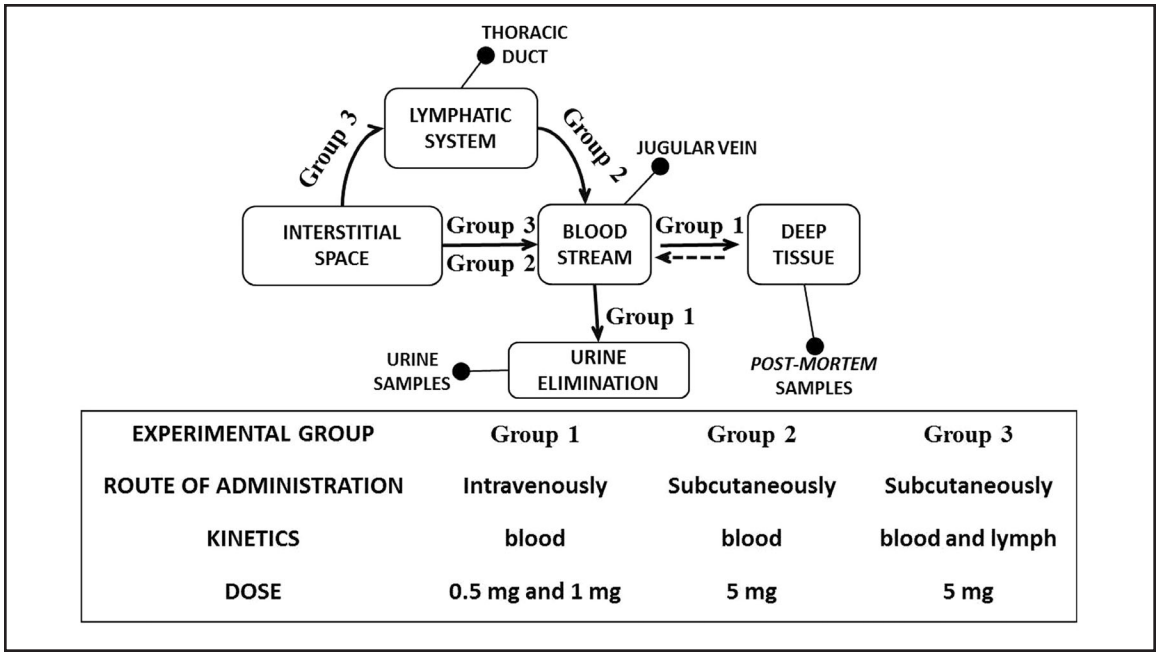
MATERIALS AND METHODS

Chemicals and Reagents

Micrurus fulvius venom was obtained from the Natural Toxins Research Center (NTRC, TAMUK, Kingsville, Texas). Antibodies for the immunoassay were produced and purified from hyperimmunized rabbits in our laboratory and biotinated using EZ-link NHS-LC-Biotin (Thermo Fisher Scientific Inc. Il. USA). Streptavidin-Peroxidase (from *Streptomyces avidinii*) was purchased from Sigma-Aldrich, México and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) was from Research Organics Inc. (Cleveland, Ohio). Fine granular quartz was from Merk (Germany).

Study Design

The study was conducted in Suffolk-Pelibuey hybrid sheep obtained from a Cuernavaca farm, and all experimental procedures were reviewed and approved by the IBT-UNAM Bioethical Committee.



EXPERIMENTAL GROUP	Group 1	Group 2	Group 3
ROUTE OF ADMINISTRATION	Intravenously	Subcutaneously	Subcutaneously
KINETICS	blood	blood	blood and lymph
DOSE	0.5 mg and 1 mg	5 mg	5 mg

Fig. 1. Experimental design to analyze the absorption, distribution and elimination of the venom administrated by subcutaneous route.

Animals were assigned to three treatment groups consisting of four sheep (*Fig. 1*). Group 1 received venom as an intravenous bolus and had blood sampled at intervals over 6 hours. Group 2 received venom SC and had blood sampled at the same intervals over 6 hours. Group 3 received venom SC and had blood sampled over 6 hours and in addition had lymph continuously collected from the thoracic duct for the 6 hours.

Surgical Techniques

Male and female adult sheep, weighing 36-60 kg were anesthetized. Anesthesia was induced with xylazine, butorphanol, and tiletamine/zolazepam and maintained with propofol and isofluorane in 100% O_2. Heart rate, blood pressure and respiration were monitored and hydration in all 3 groups was maintained with a continuous IV infusion of isotonic saline. In addition, Group 3 animals received replacement volume of saline equal to that of thoracic duct lymph removed. The jugular vein was cannulated for blood samples, and rumenotomy was performed to avoid pasture bloat. In Group 3, Evans blue dye was injected SC in the left hindlimb to assist visualization and cannulation of the thoracic duct. Cannulation was achieved by an incision into the left neck with the head recumbent to identify the thoracic duct at termination into the venous system. The duct was ligated just before entry into the vein to distend the vessel and a 22 gauge angiocath (Becton, Dickinson and Company, México) was inserted and secured with suture for collection. After 6 hours, all animals were sacrificed with an overdose of pentobarbital.

Micrurus fulvius *Venom Administration*

Solutions of *Micrurus fulvius* venom were prepared by reconstitution of lyophilized material with sterile saline to provide a final concentration of 0.5, 1.0 or 5.0 mg/ml. Intravenous injections were administered as a bolus into the saphenous vein of the right

hindlimb. In Group 1, two sheep received 1.0 mg of venom IV, and two received 0.5 mg of venom IV, in 1 ml. For Groups 2 and 3, we administered 5.0 mg SC to the left hindlimb of each sheep. The injected hindlimb was gently flexed and stretched for 30 seconds every 15 minutes throughout the 6 hours to promote lymph flow.

Serum, Lymph, Urine and Organ Sampling

After venom administration (time=0), blood samples were taken at 2, 5, 7, and 10 min, then at 5 min intervals for the next 50 min, then at 15 min intervals during the second hour, and finally every 20 min for the next four hours. Serum was separated, frozen, and maintained at -20°C until assayed.

All thoracic duct lymph was drained externally during the 6 hours and continuously collected into 15 ml tubes to measure flow for rate determination. Lymph fluid samples were centrifuged and the clear supernatant frozen, then maintained at -20°C until assayed.

All urine was collected via indwelling bladder catheter. The collecting tube was changed at 15 minute intervals and the flow recorded to calculate rate. Urine samples were centrifuged, frozen, and maintained at -20°C until assayed.

Skin samples, including subdermis, were excised from around the injection site and the corresponding contralateral site post-mortem. Skin samples were homogenized with mortar and quartz crystal technique, then extracted in 5 ml of immunoassay vehicle solution (100 mM NaCl, 0.1% gelatin, 0.05% Tween 20, in 50 mM Tris/HCl buffer at pH 8) per gram of tissue for immunoassay.

Lungs, liver, kidneys, heart and spleen were removed post-mortem, mixed in a mechanical blender and a representative sample was homogenized using an Potter-Elvehjem tissue grinder. One gram of tissue was extracted in 5 ml of immunoassay vehicle solution.

Determination of Micrurus fulvius *Venom Concentration by Sandwich Immunoassay*

Venom concentrations in lymph, serum, urine, and tissues were determined using a sandwich immunoassay as previously described (31). Briefly, 96 well plates Maxiscorp (NUNC Inc., USA) were coated with polyclonal inmunopurified antibodies from hyperimmunized rabbit serum against *Micrurus fulvius* venom. A 0.5% gelatin solution was applied as blocking solution. For detection, we used biotinated rabbit antibodies, revealed with Streptavidin-Peroxidase and ABTS. For quantification, a standard curve was established using 1:2 serial dilutions, starting with a venom concentration of 300 ng/ml. The standard curve had a lower limit of quantification of 1.3 ± 0.4 ng/ml.

Pharmacokinetic Analysis

Pharmacokinetic parameters for *Micrurus fulvius* venom in serum after IV administration were generated by fitting the serum concentration (Cs) versus time data, to a mono-exponential decay. The maximum concentration in serum (Cs_{max}) was estimated by extrapolation when t=0 (32).

The pharmacokinetic parameters after SC were estimated using a non-compartmental model. The AUC_{0-t} and $AUMC_{0-t}$ for all the experimental groups were estimated using the trapezoidal rule. Extrapolation to infinity was estimated using the last Cs measured at the time t and the terminal rate constant (λ_z) (33).

The times taken to reach the maximum concentration (t_{max}), the serum concentrations at the end of the experiment (Cz), and Cs_{max} were taken directly from analytical data.

Statistical comparisons for all parameters between treatment groups were conducted using a one-way ANOVA test and Tukey's post test in Graph Pad prism 4.0.

The fraction of the venom dose recovered in lymph was calculated as the product of the

TABLE 1p
Pharmacokinetic Parameters[a] After IV And SC Administration Of *Micrurus Fulvius* Venom

	Group 1	Group 2	Group 3	Statistical significance
Administration route	I.V.	S.C.	S.C.	
Dose (mg)	1	5	5	
Cs_{max} (ng/ml)	339 (±65)	181 (±21)	183 (±36)	G.1. ≈ G.2. ≈ G.3.
C_z (ng/ml)	1.8 (±0.1)	117 (±11)	62 (±20)	G.1. ≠ G.2. ≠ G.3.
t max (min)	-	162 (±29)	43 (±9)	G.2. ≠ G.3.
$t_{1/2}$ (min)	25.3 (±3.2)	261 (±55)	306 (±69)	G.1. ≠ G.2., G.3.; G.2. ≈ G.3.
V_D (l)	3.2 (±0.5)	-	-	
V_D (%)	7 (±0.8)	-	-	
AUC $_{0\text{-}t}$ (ng/ml * min)	13,323 (±1031)	41,050 (±3,723)	30,202 (±5,125)	G.1. ≠ G.2., G.3.; G.2. ≈ G.3.
AUMC $_{0\text{-}t}$ (ng/ml * min^2)	736,439 (±57,415)	7,401,352 (±904,639)	4,634,465 (±680,456)	G.1. ≠ G.2., G.3.; G.2. ≈ G.3.
AUC $0_{-\infty}$ (ng/ml * min)	13,743 (±1009)	85,290 (±13,640)	63,481 (±19,074)	G.1. ≠ G.2., G.3.; G.2. ≈ G.3.
AUMC $0_{-\infty}$ (ng/ml * min^2)	1,013,791 (±69,532)	41,161,824 (±11,526,922)	33,935,541(±19,087,927)	G.1. ≠ G.2.; G.1. ≈ G.3.; G.2. ≈ G.3.
CL (ml/min)	74.1 (±6)	67 (±9)	99 (±25)	G.1. ≈ G.2. ≈ G.3.
MRT (min)	75 (±7)	457 (±58)	433 (±126)	G.1. ≠ G.2., G.3.; G.2. ≈ G.3.
Vss (l)	5.7 (±0.9)	27 (±2)	36 (±6)	G.1. ≠ G.2., G.3.; G.2. ≈ G.3.
Vss (%)	12 (±1.8)	66 (±8)	71(±12)	G.1. ≠ G.2., G.3.; G.2. ≈ G.3.
MAT (min)	-	393 (±58)	369 (±126)	G.2. ≈ G.3.
F (%)[c]	-	60 (±5)	45 (±8)	G.2. ≈ G.3.
Recovered in lymph (% of dose)	-	-	25 (±3)	
Lymph flow (ml/min)	-	-	3 (±0.2)	
Total recovered (% of dose)[b]	-	60 (±5)	69 (±5)	G.2. ≈ G.3.
Recovered in urine (% of dose)	0.18 (±0.04)	0.22 (±0.12)	0.12 (±0.1)	G.1. ≈ G.2. ≈ G.3.

[a]The values shown represent the mean ± SEM

[b]Total recovered calculated for each animal, sum of the systemic availability, the cumulative percent recovered in lymph and in urine

[c]The bioavailability was calculated using the mean of AUC $_{iv}$

(≈) No significant difference between treatments ($\alpha > 0.05$)

(≠) Significant difference between treatments ($\alpha < 0.05$)

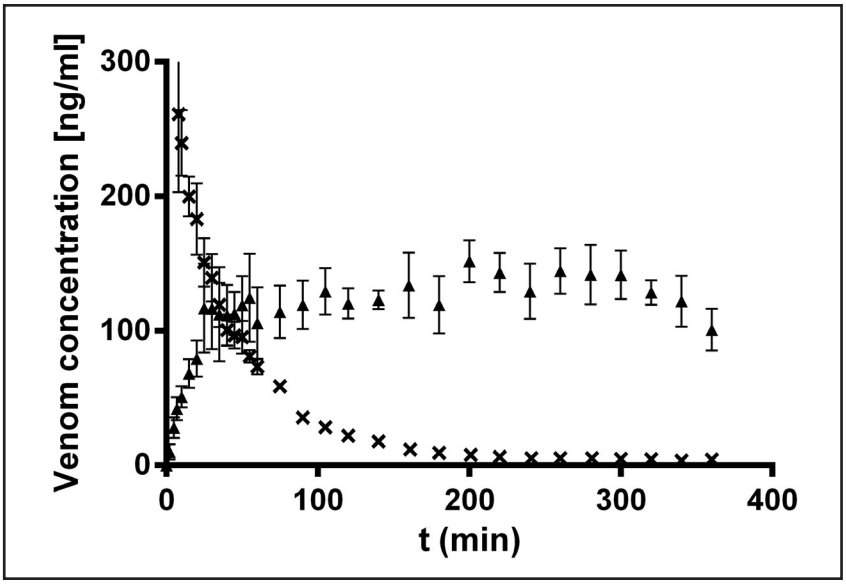


Fig. 2. Serum venom concentrations (Group 1) after the values of concentration have been corrected to 1 mg IV injection (x), and SC administration to non-cannulated sheep (Group 2) after SC injection of 5 mg of M. fulvius *venom (▲). Symbols represent the mean ± SEM for n = 4 per treatment group.*

concentration in the sample and the sample volume and was expressed as a percentage of administered dose. The cumulative percentage transported via the lymph was obtained by adding together the values of each interval. The total recovery of venom in group 3 was calculated by the sum of the amount of venom calculated in blood circulation and the cumulative fraction of dose recovered in lymph.

RESULTS

Table 1 details the pharmacokinetic parameters calculated for venom in each group. *Fig. 2* illustrates the serum venom levels, over time, in Groups 1 (IV venom injection) and 2 (SC venom injection). *Fig. 3* shows the mean serum concentrations over time following SC administration in lymph-cannulated (Group 3) and non-cannulated (Group 2) sheep. *Fig. 4* shows the cumulative percentage of venom dose collected in central lymph in Group 3, as a function of time. *Fig. 4* also illustrates a lag phase (21.9 ± 3.4 min), consistent with a delay during lymphatic flow from collecting vessels to the thoracic duct. The fraction of the injected venom that was recovered in lymph was 25 ± 3%, representing 36 ± 7% of the total accounted for in blood, lymph and urine. The highest concentration of venom found in lymph was 3,943 ± 604 ng/ml at 108 ± 25 min after venom injection. The average venom concentration in lymph during the six-hour experiment, without taking into consideration the lag phase, was 1,157 ± 327 ng/ml.

Analysis of the injection site in groups 2 and 3 confirmed the presence of 22 (± 12) µg of venom per mg of skin. Skin from the contralateral side contained negligible (0.34 ± 0.15 µg/mg). Venom in heart, lungs, spleen, and kidneys was similarly low (data not shown). The liver had the highest organ level with a total venom content of 87 ± 24 µg. Since all the organ and skin values were found to be in µg quantities, the recovered venom levels were not used in calculating the total absorbed amount.

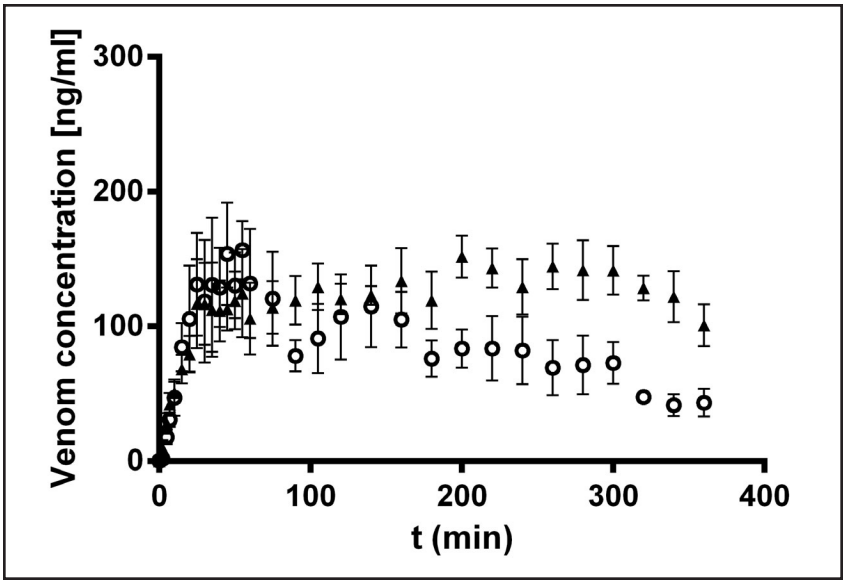


Fig. 3. Serum venom concentration following SC administration in non-cannulated [Group 2 (▲)] and lymph-cannulated sheep (Group 3 (o)) after SC injection of 5.0 mg of M. fulvius *venom. Symbols represent the mean ± SEM for n = 4 animals per treatment group.*

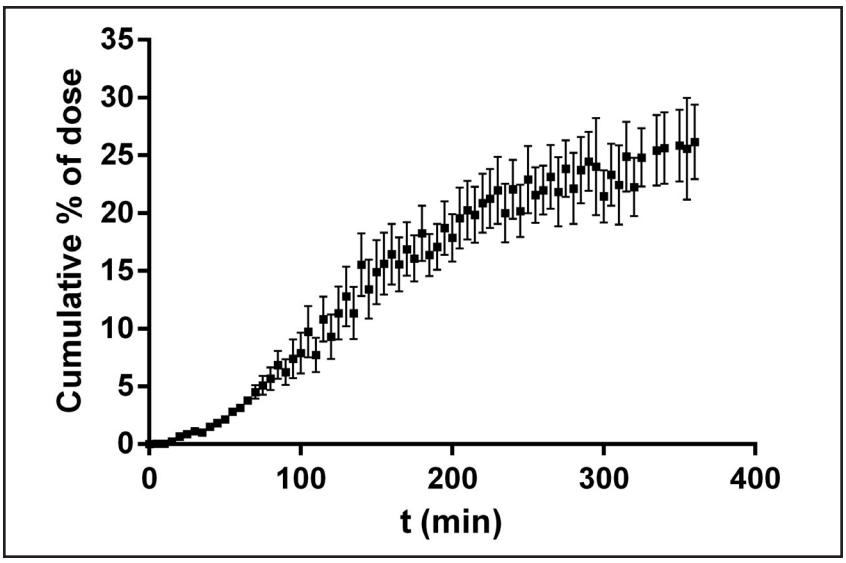


Fig. 4. Cumulative percentage of the venom dose collected in lymph-cannulated sheep (Group 3) after SC injection of 5 mg of M. fulvius *venom. Symbols represent the mean ± SEM for n = 4 animals per treatment group.*

DISCUSSION

Past models of venom pharmacokinetics have relied primarily on measurement of blood venom levels, occasionally supplemented by urine and/or selected organ venom levels. The missing parameter which we now have available is the analysis of how the venom enters, transverses, and is retained in the lymphatic system. This multi-compartment

combination approach with measurement of venom in lymph enhances our understanding of the dynamic process of how venom passes from the site of injection into the systemic circulation. The kinetic analysis of lymphatic venom absorption also enables, for the first time, an explanation of sustained venom levels in blood following envenomation.

Group 1 enabled the calculation of conventional pharmacokinetic parameters following intravenous administration. The volume of distribution (VD = 3.2 ± 0.5 l) was very close to reported estimates of blood volume in sheep (34); and the VSS (6 ± 0.9 l) remained close to VD, implying minimal distribution from blood circulation to deep tissues (Shargel et al 2005). The short half life time ($t_{1/2}$) of 25 (± 3.2) minutes, mean residence time (MRT) of 75 ± 7 minutes, and low levels detected in urine suggest that coral snake venom undergoes rapid systemic metabolism or renal degradation.

With SC administration in Group 2, conventional calculations demonstrate a more prolonged kinetic profile than following IV bolus, as expected. Rapid absorption during the first hour was followed by a steady state of venom in blood, lasting at least 5 hours following injection (*Fig. 2*). The Vss (27 ± 2l) was greater after SC than after IV administration (6 ± 0.9 l) and this finding suggests that some subcutaneously injected venom remains at the site of injection for at least several hours. This observation is in accordance with mean absorption time (MAT) of 393 ± 58 min. Together, these findings suggest that absorption from SC tissue is the limiting step for venom bioavailability, and they support the notion that the bite site serves as an ongoing venom depot (35).

The addition of a thoracic duct lymph-cannulated experimental group (Group 3) served two major purposes: first, to discover how much venom was absorbed via the lymphatic system; and second, to learn about the relative contribution of lymphatic absorption to blood venom levels. In Group 3, all lymph reaching the thoracic duct was diverted before entering the blood circulation. During the first 60 minutes after venom injection, serum levels in cannulated and non-cannulated sheep were nearly identical (*Fig. 3*), suggesting that components of venom reaching the blood stream during this time did so primarily via direct absorption into blood (or possibly with some transfer to the blood stream at the lymph-blood interface in the first draining lymph nodes). Peak serum venom levels (Cs_{max}) were comparable in the two groups (*Table 1*), but the levels diverged after the first 60 minutes, demonstrating that the impact of venom delivered by lymph to blood may include maintenance of steady state levels during the first 5 hours. Bioavailability (F, *Table 1*) was 45 ± 8% in Group 3 and 60 ± 5% in Group 2, but with the small sample size this did not reach statistical significance. Total venom recovered in blood and lymph following SC administration remained incomplete at the end of the 6-hour observation period, at 60% and 69% for Groups 2 and 3 respectively. The total amount of venom measured from all organs and urine was approximately 3% of administered dose. Because the total tissue was not recoverable from the injection site, an estimate of total remaining venom could not be obtained. The majority of the remaining venom is likely retained at or near the injection site (recovered levels at 22 µg/mg) with much smaller levels possibly remaining in the lymphatic vessels and nodes as well as a very low level in the systemic circulation prior to metabolism and elimination.

The highest concentration of venom found in lymph was more than 25 fold the highest venom concentration reached in blood. This can be explained considering the differences between lymph and blood flow rates. Since blood flow is 5,950 ± 343 ml/min (34), the venom levels are almost immediately diluted in the whole bloodstream; in contrast lymph flow rate was 3 ± 0.2 ml/min. Consequently, we can estimate an average contribution by lymph, during the course of the experiment, of 1.2 ± 0.23 ng of venom/ml

of blood/min, operating as a continuous mechanism venom supply into blood.

The immunoassay used in this study detects many different venom components, including low α-neurotoxins (around 7 kDa), neurotoxic phospholipases (13 kDa), and high molecular weight (above 13 kDa) proteins. We acknowledge that pharmacokinetics of individual venom components must vary; however, this distinction could not be demonstrated in our study. The analysis of both lymph and blood in this model suggests that the lymph pool provides a sustained inoculum of venom and associated high molecular weight products destined for entry into the bloodstream while also percolating through regional lymph nodes involved in the continuing local immune response. This highlights the unexplored importance of the lymphatic system in examining different "envenomation syndromes".

We have demonstrated that lymphatic absorption at the envenomation site plays a major role in the availability and kinetics of subcutaneously injected coral snake venom and its multiple components. Use of this combined blood and lymphatic sampling in a large animal model with the developed 3-part experimental design should provide insights into the absorption and distribution of other venoms, as well as the interaction of venoms and antivenoms and thereby shed further light on the varying clinical course of a wide variety of envenomation and also provide guidance in designing therapeutic strategies.

ACKNOWLEDGMENTS

The authors thank CONACyT (México) for financial support (project C382-08) and fellowship to Dayanira Paniagua. We also thank Felipe Olvera and Alejandro Olvera for expert technical assistance and Ricardo Mondragon for animal handling and Angélica Linares for logistics. The help of Hilda Vázquez, Roberto P. Stock and Alejandro Carbajal in the initial experiments is much appreciated.

REFERENCES

1. Kitchens, CS, LHS Van Mierop: Envenomation by the eastern coral snake (*Micrurus fulvius*). JAMA 258 (1987), 1615-1618.
2. Peterson, ME: Snake bite: Coral snakes. Clin. Tech. Small Anim. Pract. 21(2006), 183-186.
3. Norris, RL, RR Pfalzgraf, G Laing: Death following coral snake bite in the United States - First documented case (with ELISA confirmation of envenomation) in over 40 years. Toxicon. 53 (2009), 693-697.
4. Aird, SD, NJ Da Silva: Comparative enzymatic composition of Brazilian coral snake (*Micrurus*) venoms. Comp. Biochem. Physiol. 99B (1991), 287-294.
5. Vital Brazil, O: Pharmacology of coral snake venoms. Mem. Inst. Buantan. 52 (1990), 31-34.
6. Rosso, JP, O Vargas-Rosso, JM Gutiérrez, et al: Characterization of -neurotoxin and phospholipase A_2 activities from *Micrurus* venoms. Eur. J. Biochem. 238 (1996), 231-239.
7. Serafim, FG, M Reali, MA Druz-Hofling, et al: Action of *Mirurus dumerilii carinicauda* coral snake venom on the mammalian neuromuscular junction. Toxicon. 40 (2002), 167-174.
8. Dal Belo, CA, GB Leite, MH Toyama, et al: Pharmacological and structural characterization of a novel phospholipase A_2 from *Micrurus dumerilli carinicauda* venom. Toxicon. 46 (2005), 736-750.
9. Olamendi-Portugal, T, CVF Batista, R Restano-Cassulini, et al: Proteomic analysis of the venom from the fish eating coral snake *Micrurus surinamensis*: Novel toxins, their function and phylogeny. Proteomics 8 (2008), 1919-1932.
10. Dokmetjian, JC, S del Canto, S Vinzón, et al: Biochemical characterization of the *Micrurus pyrrhocryptus* venom. Toxicon. 53 (2009), 375-382.
11. Nirthanan, S, MCE Gwee: Three-finger - neurotoxins and the nicotinic acetylcholine receptor, forty years on. J. Pharmacol. Sci. 94 (2004), 1-17.
12. Norris, RL, RR Pfalzgraf, G Laing: Death following coral snake bite in the United States - First documented case (with ELISA confirmation of envenomation) in over 40 years. Toxicon 53 (2009), 693-697.
13. Pungercar, J, I Krizaj: Understanding the molecular mechanism underlying the presynaptic toxicity of secreted phospholipases A_2. Toxicon 50 (2007), 871-892.
14. Montecucco, C, JM Gutierrez, B Lomonte: Cellular pathology induced by sake venom

phospholipase A_2 myotoxins and neurotoxins: Common aspects of their mechanism of action. Cell. Mol. Life Sci. 65 (2008), 2897-2912.
15. Langgård, H: The subcutaneous absorption of albumin in edematous states. Acta Medica Scandinavica. 174 (1963), 645-650.
16. Radwanski, E, A Chakraborty, SV Wart, et al: Pharmacokinetics and leukocyte responses of recombinant human interleukin-10. Pharm Res. 15 (1998), 1895-1901
17. Charman, SA, AM Segrave, GA Edwards, et al: Systematic availability and lymphatic transport of human growth hormone administered by subcutaneous injection. J. Pharm. Science 89 (2000), 168-177.
18. Charman, SA, DN McLennan, GA Edwards, et al: Lymphatic absorption is a significant contributor to the subcutaneous bioavailability of insulin in a sheep model. Pharm. Res. 18 (2001), 1620-1626.
19. McLennan, DN, CJH Porter, GA Edwards, et al: Pharmacokinetic model to describe the lymphatic absorption of r-methu-leptin after subcutaneous injection to sheep. Pharm. Res. 20 (2003), 1156-1162
20. Kota, J, KK Machavaram, DN McLennan, et al: Lymphatic absorption of subcutaneously administered proteins: Influence of different injection sites on the absorption of dabepoetin alfa using a sheep model. DMD 35 (2007), 2211-2217.
21. Porter, CJH, SA Charman: Lymphatic transport of proteins after subcutaneous administration. J. Pharm. Science 89 (2000), 297-310.
22. Guyton, AC, JE Hall: Tratado de fisiología médica. McGraw-Hill 2001,197-211.
23. Swartz, MA: The physiology of the lymphatic system. Adv. Drug Delivery Reviews 50 (2001), 3-20.
24. Supersaxo, A, WR Hein, H Steffen: Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. Pharm. Res. 7 (1990), 167-169.
25. Ismail M, MA Abd-Elsalam, MS Al-Ahaidib: Pharmacokinetics of ^{125}I-labelled *Walterinnesia aegyptia* venom and its specific antivenins: Flash absorption and distribution of the venom and its toxin versus slow absorption and distribution of IgG, f(ab')$_2$ and f(ab) of the antivenin. Toxicon 36 (1998), 93-114.
26. Rivière, G, V Choumet, B Saliou, et al: Absorption and elimination of viper venom after antivenom administration. J. Pharmacol. Exp. Ther. 285 (1998), 490-495.
27. Lascelles, AK, B Morris: Surgical techniques for the collection of lymph from unanaesthetized sheep. Exp. Physiol. 46 (1961), 199-205.
28. Porter, CJH. GA Edwards, SA Charman: Lymphatic transport of proteins after S.C. injection: Implications of animal model selection. Adv. Drugs Del. Reviews 50 (2001), 157-171.
29. Yen, HH, JLK Wee, Snibson KJ. et al: Thoracic duct cannulation without thoracotomy in sheep: A method for accessing efferent lymph from the lung. Vet. Immunol. Immunopathol. 129 (2009), 76-81.
30. Fidler, HK, RD Glasgow, Carmichael EB: Pathological changes produced by the subcutaneous injection of rattlesnake (crotalus) venom into *Macaca mulatta* monkeys. Am. J. Pathol. 16 (1939), 355-264.
31. Boyer, LV, AA Theodorou, RA Berg, et al: Supplement to antivenom for critically ill children with neurotoxicity from scorpion stings. N. Engl. J. Med. 360 (2009), 2090-2098.
32. Shargel, L, S Wu-Pong, ABC Yu: *Biopharmaceutics & Pharmacokinetics*. McGraw-Hill, 2005, 51-59
33. AGAH working group pharmacokinetics: Collection of terms, symbols, equations, and explanations of common pharmacokinetic and pharmacodynamic parameters and some statistical functions. (2004), 3-5.
34. Upton, RN: Organ weights and blood flows of sheep and pig for physiological pharmacokinetic modelling. J. Pharmacol. Toxicol. Methods 58 (2008), 198-205.
35. Theakston, RDG: An objective approach to antivenom therapy and assessment of first-aid measures in snake bite. Ann. Trop. Med. Parasitol. 91 (1997), 857-865.

Alejandro Alagón, MD, PhD
Institute of Biotechnology / Universidad Nacional Autónoma de México
Cuernavaca, México (UNAM)
Av University # 2001,
Col. Chamilpa CP 62210
Cuernavaca, Morelos, México
alagon@ibt.unam.mx