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CARACTERIZACION BIOQUIMICA Y ELECTROFISIOLOGICA DE DOS TOXINAS DE ALACRANES MEXICANOS DE LAS ESPECIES *Centruroides limpidus limpidus* Y *Centruroides infamatus infamatus*

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN INVESTIGACION BIOMEDICA BASICA

P R E S E N T A

M. EN C. MANUEL DEHESA DAVILA

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DR. LOURIVAL D. POSSANI, DEPARTAMENTO DE RECONOCIMIENTO

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I N D I C E

RESUMEN

El veneno de los alacranes que se consideran peligrosos para el ser humano contiene un grupo heterogéneo de toxinas. Estas se han clasificado en dos grandes familias: una llamada de toxinas de cadena larga y otra la conforman toxinas de cadena corta específicas para canales de potasio (K^+). Las toxinas de cadena larga se encuentran en una mayor concentración en el veneno y ejercen su efecto fisiológico sobre los canales de sodio (Na^+) dependientes de voltaje.

Las toxinas que tienen efecto sobre mamíferos se han clasificado a su vez en toxinas de tipo α y β dependiendo del mecanismo molecular de acción sobre canales de Na^+. En esta tesis se describe la purificación y caracterización bioquímica de una nueva toxina proveniente del veneno del alacrán *Centruroides infamatus infamatus* Koch y otra del alacrán *Centruroides limpidus limpidus* Karsch. El propósito de este estudio es ahondar en el conocimiento de la relación estructura-función de las toxinas sobre canales iónicos de Na^+. Se incluyen datos sobre el efecto del veneno de *Centruroides limpidus limpidus in vivo* sobre páncreas de ratón. Del veneno del *Centruroides infamatus infamatus* se aisló el componente II.11.1 denominado toxina 1 y del *Centruroides limpidus limpidus* se aisló el componente II.9.3.4 denominado toxina 2. De ambas se obtuvo su secuencia de aminoácidos mostrando que contienen 66 residuos y entre ambas existe un 98% de homología en su estructura primaria. Se probaron ambas toxinas en experimentos de desplazamiento en membranas de cerebro de rata con toxinas marcadas con ^{125}I. El control se hizo con la toxina 2 de *C. noxius* marcada con ^{125}I, la cual se une al canal de Na^+. En ambos casos las dos toxinas nuevas desplazaron la toxina 2 de *C. noxius* indicando que reconocen al mismo sitio en el canal de Na^+. Asimismo, utilizando un sistema electrofisiológico con ovocitos de rana en los cuales se expresó el mensajero para el canal de Na^+ de músculo esquelético de

rata (clona μI2) se puso en evidencia el efecto de ambas toxinas en este tipo de canal iónico.

Concluímos que estos dos péptidos son toxinas de cadena larga que afectan a mamíferos y su efecto molecular es sobre canales iónicos de Na^+ clasificándolas como toxinas de tipo β. El efecto sobre el páncreas mostró cualitativamente alteraciones histopatológicas con cambios mínimos en la secreción exocrina.

SUMMARY

The venom of scorpions considered to be poisonous to human beings contain a heterogeneous group of toxins. These have been clustered in two big families of peptides, one the so-called long chain toxins and the other the short chain toxins. The long chain toxins are found in a greater concentration and exert their physiological effect on the Na^+ channels which are voltage dependent. In turn, the toxins specific to mammals have been classified in toxins class α and β depending on their effect on the Na^+ channel.

This dissertation describes the purification and the biochemical characterization of two toxins isolated from the venom of the scorpions *Centruroides infamatus infamatus* Koch and *Centruroides limpidus limpidus* Karsch. The purpose of the study is to contribute to the knowledge of the structure-function relationship of the toxins actives upon Na^+ ionic channels. Results concerning the effect of the venom of *Centruroides limpidus* on mouse pancreas are included. From the venom of the *Centruroides infamatus* the component II.11.1 denominated toxin 1 was isolated. From the *Centruroides limpidus* venom the component II.9.3.4. denominated toxin 2 was isolated. For both, toxin 1 and 2, their amino acid sequences were obtained. They contain 66 amino acid residues and showed a 98% identity on their primary structure. Both toxins were tested in binding-displacement experiments using rat brain synaptosomal membranes and radiolabeled toxin 2 from *C. noxius*, another well characterized Na^+ channel toxin. Toxin Cii 1 and Cll 2 displaced binding of ^{125}I-Cn toxin 2 with very similar affinities and kinetics. Using a frog oocyte expression system with the Na^+ channel clone μI2 from rat skeletal muscle it was shown that both toxins affect ion permeability in this type of channel.

We conclude that the novel toxins described here are long-chain peptides, specific for mammalian Na^+ channels, and modified the function of these channels in a similar way as that described for toxins of the class β "beta". The effect on the pancreas showed qualitatively hystopathologic alterations with minimal effects on the exocrine secretion.

PREFACIO

Esta tesis recopila los resultados experimentales obtenidos durante el curso de Doctorado como alumno de la UACPyP en el Instituto de Biotecnología de la UNAM, comprendiendo cuatro publicaciones como primer autor.

El trabajo realizado se presenta de la siguiente manera:

1.- Artículo sometido como primer autor:
"DEHESA-DÁVILA M., RAMIREZ A. N., ZAMUDIO Z. F., GURROLA G. B., LIEVANO A., DARZON A. Y POSSANI L. D. Structural and functional comparison of toxins from the venom of the scorpions *Centruroides infamatus infamatus*, *Centruroides limpidus limpidus* and *Centruroides noxius*", sometido a la revista Comparative Biochemistry and Physiology.

Este artículo contiene los resultados de una serie de experimentos comparativos, tanto de la parte estructural como funcional, de toxinas provenientes de tres especies diferentes de alacranes mexicanos.

Las dos publicaciones siguientes contienen: una, los resultados obtenidos sobre el aislamiento de una toxina del veneno del alacrán *Centruroides infamatus infamatus* y su efecto sobre los canales de Na^+ en células nerviosas. El otro artículo versa sobre el problema del alacranismo en México abordando aspectos epidemiológicos y centra su atención en el uso clínico del antisuero antialacrán, el cual muestra su utilidad y eficiencia. Este artículo fue elaborado por invitación del Dr. Alan Harvey, editor de la revista Toxicon al Dr. L. D. Possani (anexos 1 y 2).

2.- DEHESA-DÁVILA M., MARTIN B. M., NOBILE M., PRESTIPINO G. Y POSSANI L. D. (1994) Isolation of a toxin from *Centruroides infamatus infamatus* Koch scorpion venom that modifies Na' permeability on chick dorsal root ganglion cells. Toxicon 32:1487-1493.

3.- DEHESA-DÁVILA M. Y POSSANI L. D. (1994) Scorpionism and Serotherapy in Mexico. Toxicon 32; 1015-1018.

Finalmente, se anexa otra publicación como primer autor en donde se desglosa la distribución de las especies venenosas de alacranes en nuestro pais, sus caracteristicas epidemiológicas, cuadro clinico, primeros auxilios y tratamiento médico (anexo 3).

4.- DEHESA-DÁVILA M., ALAGÓN C. A. Y POSSANI L. D. (1994) Clinical toxicology of scorpion stings. En: CRC Handbook of Human Toxicology Series, Editores Jurg Meier y Julian White (en prensa).

Además se presentan algunos resultados no publicados sobre la secreción exocrina de páncreas y el desarrollo de una pancreatitis aguda en ratones, mostrada por la determinación de amilasa sérica y estudios de histopatología con microscopía de luz. Estos experimentos se llevaron a cabo durante una estancia en el laboratorio del Dr. Paul L. Fletcher Jr., Departamento de Microbiología e Inmunología de East Carolina University, Greenville, Carolina del Norte, USA. También, durante el desarrollo de esta tesis se publicó un capítulo de libro titulado: Protección contra el alacranismo en Vacunas, Ciencia y Salud, (editores Alejandro Escobar Gutiérrez, José Luis Valdespino Gómez y Jaime Sepúlveda Amor) editado

por la Secretaría de Salud, Subsecretaría de Coordinación y Desarrollo (1992) de los autores Lourival D. Possani, Emma S.A. Calderón, Timoteo Olamendi P., Manuel Dehesa-Dávila y Georgina B. Gurrola, del cual no se incluye copia.

I INTRODUCCION

A) GENERALIDADES

El alacranismo en México es un problema de salud pública que afecta a la población tanto del medio rural como del medio urbano con 200,000 casos de envenenamiento y 310 muertes por año, lo cual constituye las cifras más elevadas de morbi-mortalidad (Dehesa-Dávila y Possani, 1994).

Las especies tóxicas de alacranes de importancia médica tienen una amplia distribución en el mundo. Se localizan al Norte de Africa, Medio Oriente e India y pertenecen a los géneros *Androctonus, Leiurus, Buthus, Buthotus y Heterometrus.* En el continente americano, los géneros tóxicos son el *Centruroides* en el sur de los Estados Unidos y México y el *Tityus* localizado principalmente en Trinidad y Tobago, Venezuela y Brasil. Todos estos géneros pertenecen a la familia Buthidae (Hoffmann, 1932; Balozet, 1971).

En México, los estudios taxonómicos y biogeográficos más completos sobre los alacranes mexicanos fueron realizados por Hoffmann en la década de los años treintas (1932, 1935, 1939). Estos son arácnidos que pertenecen al orden Scorpionida, familia Buthidae, género *Centruroides*, con la existencia de 32 especies y subespecies.

En el anexo 2 y 3 se detalla la distribución geográfica de los alacranes más venenosos y de importancia médica así como el impacto epidemiológico del alacranismo.

B) RELACIÓN ESTRUCTURA FUNCIÓN DE NEUROTOXINAS

El estudio del veneno de alacrán se inició en respuesta al problema médico y de salud pública que representaba el envenenamiento producido por la picadura de este animal. En nuestro país fue el Dr. Maximino Ruiz Castañeda quien, en el año de 1933 durante una estancia en la Universidad de Harvard, describió el método para inmunizar equinos y obtener un antisuero que neutralizara los efectos del veneno (Ruiz Castañeda, 1933). Con ello sentó las bases de lo que hoy conocemos como seroterapia y comúnmente es llamado suero ó antisuero antialacrán. En la actualidad, el aspecto más relevante de estos estudios es el uso de toxinas purificadas para la investigación de los mecanismos moleculares que ejercen sobre membranas excitables y el aislamiento y caracterización de diferentes canales iónicos (Catterall, 1979; Possani, 1982, Hille, 1992).

El veneno de alacrán de la familia Buthidae está compuesto en su mayoría por polipéptidos neurotóxicos y pequeños péptidos, de función aún desconocida (Zlotkin, 1978; Possani, 1980). En general, el veneno es de apariencia lechosa, de color opalescente y con un pH de alrededor de 7.0. Los polipéptidos básicos con peso molecular alrededor de 7,000 daltones y con actividad neurotóxica están contenidos en la parte soluble y consisten en su mayor parte de una mezcla de proteínas y péptidos de pesos moleculares diferentes y en menor cantidad lípidos, nucleótidos, sales inorgánicas y aminoácidos libres (Possani, 1983).

El grupo encabezado por el Dr. Possani ha tenido como programa de investigación el estudio del veneno de alacrán, para ello se han purificado y caracterizado toxinas tanto del género *Centruroides* como de *Tityus*, éstas son: 11 toxinas de *C. noxius* (Possani *et al.*, 1981a:, Possani *et al.*, 1981b:, Dent, M.A.R., 1982:, Possani *et al.*, 1982:, Zamudio *et al.*, 1992), 5 de *C. limpidus tecomanus* (Possani *et al.*, 1980: Ramírez *et al.*, 1988: Martin *et al.*, 1988) una de *C. elegans* (Possani *et al.*, 1978: Ramírez *et al.*, 1981), 6 de *Tityus serrulatus* (Possani *et al.*, 1977, 1981c, 1982, 1985 y 1991) 10 de *Tityus bahiensis* (Possani *et al.*, 1992), 6 de *C. limpidus*

limpidus (Ramírez *et al.*, 1988, Ramírez *et al.*, 1994) y una de *C. infamatus infamatus* (Dehesa-Dávila *et al.*, 1994).

Las toxinas a mamíferos que afectan el canal de sodio (Na^+) ocupan un primer lugar puesto que son las más abundantes en el veneno del género *Centruroides*. El tamaño de la cadena polipeptídica es muy semejante, entre 65 y 66 residuos de aminoácidos y la homología en su estructura primaria varia entre un 62 y 98% (ver anexo 1). Además, existen ejemplos de semejanza en su plegamiento y estabilización de su estructura terciaria como es el caso de la toxina variante 3 de *C. sculpturatus* (Meves *et al.*, 1986, Gurrola *et al.*, 1994). Estas toxinas son llamadas de cadena larga.

Otro grupo de toxinas lo componen un tipo de toxinas que actúan sobre el canal de potasio (K^+). Estas son toxinas de cadena polipeptídica más corta con 37 a 39 residuos de aminoácidos y se les ha denominado de cadena corta. Este grupo lo encabeza la Noxiustoxina del *C. noxius* (Possani *et al.*, 1982). En el axón gigante de calamar su efecto disminuye la permeabilidad del canal de K^+ en forma reversible e independiente del voltaje. Esto no afecta al canal de Na^+ (Carbone *et al.*, 1982). Este tipo de toxinas no es exclusiva de esta especie sino que se han encontrado en otras especies del mismo género como es el caso de *C. limpidus limpidus, C. elegans* (Carbone *et al.*, 1983). Recientemente, otras toxinas específicas para canales de K^+ fueron aisladas y caracterizadas en el veneno de *C. limpidus limpidus* (Martin *et al.*, 1994).

Una diferencia sustancial entre estos dos grupos es su estructura primaria. Para las toxinas que afectan el canal de Na^+, la cadena polipeptídica tiene alrededor de 65 a 66 residuos de aminoácidos y las de K^+ tiene entre 37 y 39 aminoácidos. Estas diferencias dan como resultado una función diferente, si bien la estructura tridimensional de ambas familias de toxinas presenta una región de α-hélice y tres regiones de lámina plegada β muy semejantes.

Las toxinas específicas para canal de Na^+ tienen un puente disulfuro de más y presentan cuatro segmentos de lámina plegada β beta (Ménez et al., 1992). Otro grupo de toxinas son aquellas que afectan a larvas de moscas, grillos, cucarachas y otros insectos. Sin embargo, en este grupo hay toxinas que tienen una cadena larga y otras de cadena corta (Pelhate y Zlotkin, 1982). Como ejemplo tenemos la toxina contra insectos de *A. australis* con 70 aminoácidos (Darbon *et al.*, 1982) y la toxina corta I-1 del *B. eupeus* de 37 aminoácidos (Zhdanova *et al.*, 1978).

C) CANALES IONICOS

Los canales iónicos son proteínas de las membranas celulares compuestas por varias cadenas polipeptídicas, cuya función es controlar el paso de iones a través de las células. Así, determinan la polaridad (diferencia de potencial de la membrana) y gran parte de la actividad eléctrica de las células excitables como nervios, músculos y otros tejidos. Ellos producen y transducen señales eléctricas en las células vivas. Recientemente con el advenimiento de nuevas técnicas de bioquímica, farmacología y biofísica de membrana, se ha podido estudiar mejor los canales iónicos y reconocer un interés creciente por ellos en otras células que no son del tejido nervioso. Los espermatozoides, leucocitos y glándulas endocrinas requieren de canales para funcionar. Una membrana celular excitable única puede tener de 5 a 10 tipos de canales y su genoma probablemente codifique para más de 50 (Hille, 1992).

Los canales iónicos son verdaderos poros macromoleculares en la membrana celular y son los elementos excitables fundamentales en las membranas de las células. En forma análoga, los canales iónicos son como la señal eléctrica al nervio, músculo y sinapsis o como las enzimas al metabolismo.

La excitación y la señal eléctrica en el sistema nervioso involucra el movimiento de iones a través de los canales iónicos. Los iones de Na^+, K^+,

Ca^{++} y Cl^{-} son los responsables de estas acciones. Cada canal puede considerarse como una molécula excitable que responde en forma específica a cierto estímulo que puede ser un cambio de potencial de membrana, un neurotransmisor o una deformación mecánica (Hille, 1992).

En la naturaleza no sólo se encuentran neurotoxinas que bloquean selectivamente los canales iónicos sino que también modifican su cinética de apertura. De ellas tenemos ejemplos que incluyen toxinas peptídicas provenientes del veneno de alacranes, en los tentáculos de los celenterados de nematocistos, toxinas alcaloides secretadas por ranas tropicales y otras sustancias insecticidas liposolubles de algunas plantas (Lazdunski *et al.*, 1986). Estas actúan sobre el canal de Na^{+} o el de K^{+} incrementando su probabilidad de apertura o dejándolo abierto. Causan dolor y muerte promoviendo el disparo repetitivo o una despolarización constante del nervio o músculo e inducen arritmias cardiacas. Estas toxinas son interesantes puesto que finalmente son marcadores bioquímicos específicos y medios químicos para activar los canales de Na^{+}. Un simple tratamiento químico o aún una modificación del contenido iónico en el medio se puede utilizar para modificar su función. Iones divalentes afectan la dependencia al voltaje. Hay tres clases o tipos mayores de modificaciones de apertura: (1) prevención de la inactivación (2) promoción de la activación en reposo y (3) cambio en la dependencia de voltaje de todos los procesos de apertura. Mediante experimentos de electrofisiología se verificó que las toxinas tienen diferentes efectos y esto ha permitido agruparlas en varias clases (Watt *et al.*, 1984):

a) Toxinas de alacrán del Viejo Mundo: Estas disminuyen la inactivación del canal de Na^{+} sin alterar su activación. Su efecto es dependiente de voltaje y es disminuído por la despolarización de la membrana. Las toxinas de este tipo son llamadas toxinas α. Ejemplos de estas toxinas se encuentran en el veneno de los alacranes:

- ***Leiurus quinquestriatus*** **(Norte de África)**
- ***Buthus eupeus; Buthus tamalus*** **(Asia)**
- ***Androctonus australis*** **(Norte de África)**

b) Toxinas de alacrán del Nuevo Mundo: Ocasionan una despolarización transitoria del canal de Na^+ induciendo un corto circuito en la dependencia de voltaje en el mecanismo de la activación. Este tipo de toxinas son denominadas toxinas β. Ejemplos de estas toxinas se encuentran en los venenos de:

- ***Centruroides sculpturatus; Centruroides suffusus***
- ***Tityus serrulatus***

c) La toxina gamma (γ) modifica tanto la activación como la inactivación del canal de Na^+ y tiene la constante de afinidad más baja reportada hasta ahora (Jonas *et al.*, 1986).

- ***Tityus serrulatus*** **(TiTx γ gamma).**

Entre las toxinas de alacranes que actúan sobre el sistema neuromuscular de vertebrados están las que modifican a canales de sodio sensible a voltaje. Estos canales son responsables de la fase rápida de despolarización del potencial de acción en nervio, músculo y células cardiacas. La apertura y cierre de éstos es controlado por activación e inactivación, dos procesos distintos dependiendo del potencial de membrana y del tiempo.

Una variedad de neurotoxinas ha mostrado modificar en forma especifica el funcionamiento normal del canal de Na^+ permitiendo conocer su estructura y función las cuales pueden actuar en cuatro sitios receptores diferentes. La asignación de las toxinas a los sitios de acción en forma separada se ha basado por interacciones sinergísticas y competitivas como se analiza en estudios de pegado (Catterall *et al.*, 1986) (Cuadro 1).

Se han definido cuatro sitios de pegado de las neurotoxinas por medio de estudios de electrofisiología, flujos con iones radioactivos y estudios de ligandos (Couraud *et al.*, 1982).

En el sitio 1 las toxinas se pegan a un receptor común el cual se piensa está localizado cerca de la apertura extracelular del canal. El sitio 2 probablemente esté ubicado en una región del canal que involucra una activación e inactivación dependiente del voltaje. El sitio 3 está localizado en la parte del canal que acopla la activación e inactivación. El sitio 4 fija a las llamadas toxinas β de los alacranes del género *Centruroides* las cuales modifican la activación en vez de la inactivación.

CUADRO 1

SITIOS RECEPTORES DE NEUROTOXINAS EN EL CANAL DE SODIO

SITIO	NEUROTOXINA	EFECTO FISIOLÓGICO
1	Tetrodotoxina Saxitoxina Geografutoxina	Inhibe el transporte iónico
2	Veratridina Batracotoxina Grayanotoxina Acontina	Activación persistente
3	Toxinas α de alacranes del Norte de África Toxinas de anémona de mar	Inactivación lenta
4	Toxinas β de alacranes Americanos	Realza la activación

D) EFECTO SOBRE PÁNCREAS

Freire-Maia *et al.*, (1994) ha reportado diversos efectos del veneno de *Tityus serrulatus* en rata tales como: arritmias cardiacas, hipertensión arterial, edema pulmonar, arritmias respiratorias y un aumento excesivo en las secreciones salivales, gástricas y pancreáticas. Estos efectos están mediados por la estimulación del sistema nervioso periférico.

Bartholomew (1970) fue de los primeros en llamar la atención sobre el efecto del envenenamiento por *Tityus trinitatis* en páncreas. Este alacrán ocasiona como parte del cuadro clínico una hiperamilasemia, dolor abdominal y pancreatitis aguda, la cual puede evolucionar en forma rápida a una pancreatitis necro-hemorrágica . Se habían propuesto básicamente dos teorías para explicar el mecanismo de acción. Una contempla que el efecto del veneno es a través de una hiperestimulación por medio de la acetilcolina como neurotransmisor a nivel de las terminaciones nerviosas del tabique interlobular en el páncreas (Singh *et al.*, 1978) y la otra postula que el veneno afecta el esfínter de Oddi contrayéndolo y así obstruir la salida de la secreción pancreática. Al aumentar la presión intraductal y haber reflujo de la secreción, esta es la que ocasiona la inflamación del páncreas traduciéndose en pancreatitis (Bartholomew *et al.*, 1977). Dependiendo de la agresión puede ser una forma edematosa o evolucionar a una forma necro hemorrágica.

Possani *et al.*, (1991) y Fletcher *et al.*, (1992) corroboraron que tanto el veneno total como las toxinas III-8, III-10 y IV-5 del *Tityus serrulatus* actúan como secretagogos en lóbulos pancreáticos.

La toxina γ de *Tityus serrulatus* es un bloqueador del canal de Na^+ dependiente de voltaje en el axón gigante de calamar (Jonas et al., 1986) y en canales de Na^+ de corazón (Yatani *et al.*, 1988, Kirsh *et al.*, 1989). Por otro lado los dos tipos de canales descritos en las células acinares de páncreas han sido el canal de Ca^{2+} el cual es activado en forma no selectiva por cationes monovalentes (permeable al K^+) y el canal de Ca^{2+}

activado en forma selectiva por K^+. Los canales de Na^+ activados por voltaje han sido estudiados en las células secretoras de insulina (Rorsman *et al.*, 1986) mas no han sido investigados o demostrados en las células acinares. Por ello, esta nueva evidencia abre la posibilidad de que estas toxinas actúen a nivel de la membrana celular, sin embargo que actúen o no en forma directa sobre estas células no desmerece que sean estudiadas para elucidar el mecanismo a través del cual pueden afectar los procesos de secreción exócrina ó endócrina en páncreas (Fletcher *et al.*, 1992).

II PRESENTACION DE LA TESIS

El trabajo que presentamos está ubicado dentro del contexto de estudio sobre la relación estructura-función de las toxinas del veneno de alacranes mexicanos que afectan y son especificas para el canal de Na^+. Describimos en forma breve su marco teórico y sus antecedentes. Los resultados se muestran en forma de artículo científico los cuales ya han sido aceptados, salvo el primero que está sometido a revisión.

Dados los antecedentes expuestos sobre el efecto fisiopatológico y considerando los trabajos efectuados por el grupo del Dr. Fletcher con el veneno y las toxinas del alacrán *Tityus serrulatus* y *Tityus trinitatis* (Possani *et al.*, 1991, Fletcher *et al.*, 1992, Fletcher *et al.*, 1994) en páncreas, nuestro proyecto de tesis se centró en la purificación y caracterización bioquímica y electrofisiológica de las toxinas mayoritarias de los alacranes *Centruroides infamatus infamatus* y *Centruroides limpidus limpidus*, las cuales son el objeto del presente trabajo. Sin embargo, presentamos algunos datos de secreción y microscopía de luz utilizando veneno de *Centruroides limpidus limpidus*, puesto que hacían parte de los objetivos presentados al inicio del proyecto de tesis doctoral.

III STRUCTURAL AND FUNCTIONAL COMPARISON OF TOXINS FROM THE VENOM OF THE SCORPIONS Centruroides infamatus infamatus, Centruroides limpidus limpidus AND Centruroides noxius.

Structural and Functional comparison of toxins from the venom of the scorpions *Centruroides infamatus infamatus*, *Centruroides limpidus limpidus* and *Centruroides noxius*

by

Manuel Dehesa-Dávila, Angelina N. Ramírez, Fernando Z. Zamudio, Georgina Gurrola-Briones, Arturo Liévano, Alberto Darszon, and Lourival D. Possani*

Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Apartado Postal 510-3, Cuernavaca, Morelos 62271, México.

* To whom correspondence should be addressed.
Instituto de Biotecnología-UNAM
Avenida Universidad, 2001
Apartado Postal 510-3
Cuernavaca, Morelos 62271, México
Tel. (--52 73) 171209
Fax: (--52 73) 172388
Electronic mail: possani@pbr322.ceingebi.unam.mx

Running title: Two novel scorpion toxins.

Abbreviations: *C*, *Centruroides*; Cii, *Centruroides infamatus infamatus* Koch; Cll, *Centruroides limpidus limpidus* Hoffmann; Clt, *Centruroides limpidus tecomanus*; Cn, *Centruroides noxius* Hoffmann; Css, *Centruroides suffusus suffusus*; CMC, carboxymethyl-cellulose; E_H, holding potential, IC_{50}, inhibition concentration 50%; *l*, *limpidus*; mol-wt, molecular weight; RC-toxin, toxin reduced and carboxymethylated; T, *Tityus*.

ABSTRACT

1. Two novel toxins containing 66 amino acid residues each were isolated from the venom of the scorpions *Centruroides infamatus infamatus* and *Centruroides limpidus limpidus*, respectively. Their full amino acid sequences were determined.

2. Comparison of primary structures showed that they share 97% similarity among themselves and 83% to that of toxin 2 from *Centruroides noxius*.

3. The three toxins studied compete with each other for the same binding sites on membranes prepared from rat brain synaptosomes, suggesting that they are all ß-scorpion toxins.

4. Toxin action was assayed into the μI-2 rat skeletal muscle Na^+ channel heterologously expressed into *Xenopus* oocytes. All three toxins block this Na^+ channel in a similar fashion, without affecting inactivation, and showed IC_{50} values in the micromolar concentration range.

Key words: amino acid sequence, *Centruroides infamatus*, *Centruroides limpidus*, *Centruroides noxius*, Na^+ channel, scorpion toxins, oocyte expression.

INTRODUCTION

In Mexico the only scorpions that represent a life hazard to humans belong to the genus *Centruroides*, family Buthidae (review by Dehesa-Dávila and Possani, 1994a). Among the most important species are *Centruroides limpidus limpidus* Koch (Cll) from the states of Morelos and Guerrero, and *Centruroides infamatus infamatus* Hoffmann (Cii) from the state of Guanajuato. These two species are responsible for severe human envenomation (Dehesa-Dávila, 1989, Alagón *et al.*, 1988, Ramírez *et al.*, 1994). For example, *C. infamatus* causes at least 10,000 cases of envenomation per year in the city of Leon, Guanajuato, (Dehesa-Dávila, 1989, Dehesa-Dávila and Possani, 1994a). However, the venom of *Centruroides noxius* Hoffmann (Cn) is the most toxic of all the Mexican scorpions (Dent el al., 1980), but medically less important, because *C. noxius* does not cohabit with humans. *C.l.limpidus* and *C.i.infamatus* very often are inside of houses and gardens increasing the probability of human accidents (Dehesa-Dávila *et al*, 1994a, 1994b).

The venom from *C.noxius* is the only well characterized (Dent *et al.*, 1980, Carbone *et al*, 1982, 1987; Possani, 1984; Zamudio *et al*, 1992; Becerril *et al*, 1993, Valdivia *et al.*, 1994). A preliminary characterization of toxic peptides from *C.l.limpidus* venom was made by Tato *et al* (1978), followed by Alagón *et al* (1988) which reported the isolation and functional characterization of two toxins from this venom, one of which has been fully sequenced (Ramírez *et al*, 1994). Three novel toxins from *C.l.limpidus* were recently reported (Martin et al., 1994; Lebreton *et al.*, 1994). However, the venom of *C.i.infamatus* is much less studied (Dehesa-Dávila, 1990, and Dehesa-Dávila *et al.*, 1994b).

The present communication describes the isolation and full amino acid sequence of two novel toxins, each one corresponding to the major toxic component of *C.l.limpidus* and *C.i.infamatus* venoms, respectively.

Both toxins displace with high affinity the *Cn* toxin-2 bound to rat brain synaptosomes, suggesting that the two of them belong to the ß-class family of Na^+ channel-specific scorpion toxins (Valdivia *et al.*, 1994). Additionally, both decrease the peak Na^+ currents carried out by the µI-2 rat skeletal muscle channel expressed in *Xenopus laevis* oocytes, without affecting the channel inactivation.

MATERIALS AND METHODS

Source of venom

C.i. infamatus scorpions were collected by us in the city of Leon (Guanajuato state), while *C.l. limpidus* and *C. noxius* scorpions were collected, respectively, in the region of Iguala (Guerrero State) and Tepic (Nayarit State). The venoms were obtained by electrical stimulation of anaesthetized animals as previously described (Dent et al., 1980). Water solubilized venom was centrifuged at 16,000 *g* for 15 min in a Sorvall SS-34 rotor. The supernatant was freeze-dried and stored at -20°C until used.

Materials

Only analytical grade chemicals and reagents were used. Sephadex G-50 (fine) was from Pharmacia Fine Chemicals (Uppsala, Sweden); Carboxymethyl-cellulose (CMC-32) was from Whatman (Clifton, NJ, USA). Reagents for amino acid analysis and sequence were purchased from Millipore Co. (Bedford, MA) and Beckman Co. (Palo Alto, CA, USA), as described by Zamudio *et al.*, 1992. Solvents for high performance liquid chromatography (HPLC) were from Pierce Chemical Co. (Rockford, IL). Gentamycin was from Sigma Co. (St. Louis, MO, USA). Water double distilled over quartz was used all through the purification and characterization steps.

Lethality test

The mouse lethality of various protein fractions was observed after intraperitoneal injection of different amounts of protein (usually from 10 to 40 μg) in 0.2 - 0.4 ml saline or buffer solutions, into adult mice (strain CD1), weighing from 20-25 g each. To define the toxicity of various chromatographic components, three designations were used: "Lethal" means that the component at the dose injected was enough to kill the tested mouse; "Toxic" means that the mouse showed any of the following symptoms: excitability, salivation, temporary paralysis of rear limbs, dyspnea, but recovered within 20 hr after injection; and, "Non-toxic" means normal behaviour similar to injection of 0.9% NaCl or buffer solutions.

Purification procedures

Toxin 2 from *C. noxius* was purified as described previously (Zamudio *et al.*, 1992). The two novel toxins were purified as follows: the soluble venoms were applied independently to a Sephadex G-50 (fine) column (200 cm x 1 cm). The toxin containing tubes were pooled (fraction number II) and chromatographed individually on a CMC-32 column, equilibrated and run in 20 mM ammonium acetate buffer at pH 4.7. The major toxic fraction obtained (II.11 from Cii and II.9 from Cil) was subsequently

60 min at room temperature. The reaction was stopped by addition of 5 ml of cold binding medium. The membranes were immediately collected on glass fiber filters (Whatman GF/B) under vacuum and washed three times with cold binding medium. The filters were dried and counted in a gamma counter. All values are an average of at least triplicate experiments.

μI-2 Na^+ channel functional expression

Oocyte positive frogs were obtained from Nasco Biologicals, Inc. (Fort Atkinson, WI), and maintained at 20 - 25 °C. They were fed three times a week with frog bristles (Nasco) during 2 hs. After feeding the frogs the aquarium was washed and all the water replaced. Oocyte isolation and selection was according to established protocols (Sumikawa et al., 1989). The oocytes were kept on ND-96 medium (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$ and 1 Cl_2Mg), supplemented with 50 μg gentamycin/ml at 19°C until use.

The rat skeletal muscle Na^+ channel clone μI-2 (Trimmer et al, 1989; kindly provided by Dr. Gail Mandel) was linearized with the restriction enzyme Not-I. The transcription was carried out at 37°C with the T7 RNA polymerase (Riboprobe kit; Promega, Madison, WI), in the presence of 500 μM of the capping analog m'G(5')ppp(5')G , according to the supplier's suggested protocol. Stage V and VI oocytes (Dumont, 1972) were selected and injected with 50 nl of μI-2 mRNA (10-50 ng), and were incubated for two to three days in ND-96 medium at 19°C. Whole-cell currents were recorded under voltage-clamp with a two microelectrode voltage-clamp (Clampator-1, Dagan Corp., Minneapolis, MN), at 20-22°C in ND-96. The microelectrodes, electrically connected to the headstages with Ag/AgCl wires, were filled with KCl 3M, and capacitative transients and leakage currents were subtracted both analogically and by the P/4 procedure (Bezanilla and Armstrong, 1977).
Ionic currents were filtered at 5kHz (-3db point, 4 pole Bessel filter), digitized on-line at 20 kHz with a Digidata 1200 data acquisition system using the pClamp 6.0 (Axon Instruments Inc.; Foster City, CA) software, and analyzed off-line. The recording chamber had a 250 μl volume, and control records were always obtained in ND-96. Toxins were diluted to 1 ml ND-96, and the oocyte chamber perfused with them. The currents were assayed 20 min later. IC_{50}'s were calculated according to:

$$IC_{50} = [T]\ (1-F_B)/F_B$$

where [T] corresponds to the toxin concentration, and F_B is the fraction of the peak current blocked at -10 mV test potential. We used this test potential to survey scorpion toxin effects because it is near the peak of the I-V relationship. In all cases the holding potential (E_H) was -70mV, and toxin concentration was either 0.5 or 1 μM.

purified by a couple of additional steps, either with ion exchange chromatography on CMC-32 resins at pH 6.0 or by high performance liquid chromatography (HPLC) in the system indicated below (see figure legends for details).
Before use the columns were always equilibrated with the initializing buffers. Columns were run at room temperature (22-25°C). Whenever necessary the fractions were dialysed against the appropiate buffers prior to rechromatography using a Spectrapor 3 M dyalisis membrane (Spectrum Medical Industries, Los Angeles, CA) The purity of the fractions was followed by polyacrylamide gel electrophoresis (PAGE) containing urea as described by Reisfeld et al.(1962). Protein content of venoms and chromatographic fractions were estimated at 280 nm, assuming that one unit of absorbance is equal to a protein content of 1 mg/ml.

Chemical characterization of the toxins

Amino acid analysis of samples were conducted in a Beckman 6300E analizer, after acid hydrolysis in 6 N HCl, for 24 hr at 110 °C in tubes sealed under vaccum. The amino acid sequence was performed in a ProSequencer apparatus from MilliGen/Biosearch, Division of Millipore. Pure toxins were sequenced initially as such for the N-terminal portion, but most sequence data were obtained with reduced and carboxymethylated samples (RC-toxin) prepared as described (Martin *et al.*, 1988). Samples containing 70 to 150 µg peptide were processed at each time. Usually 1.0 nmol of RC-toxin, or cleaved peptides (see below) were attached to an Immobilon-CD membrane (Millipore, place, U.S.A.) for sequence determination.

Enzymatic digestion and peptide separation

Enzymatic cleavage of RC-toxins was conducted by digestion with *Staphylococus aureus* V8 protease (Miles laboratories, Stoke Poges, Bucks, U.K.) and Trypsin (Boehringer Mannheim) as described previously (Possani *et al.*, 1985). The resulting peptides were separated by HPLC, as indicated in the figure legends, and sequenced.

Toxin binding to rat brain membranes

Rat brain membranes (fraction P3) were obtained according to Caterall *et al.*, (1979). *C. noxius* toxin 2 was iodinated with ^{125}I by the lactoperoxidase method (Morrison and Bayse, 1970). ^{125}I-labelled toxin was purified using a rapid filtration assay in a Sephadex G-10 column (7 x 0.5 cm). The reaction buffer for the binding assay consisted (in mM): 140 choline cloride, 5 KCl, 1.5 CaCl, 0.8 $MgCl_2$, 20 Tris-HCl, pH 7.4 and 0.1% bovine serum albumin. The binding reaction was initiated by addition of brain membranes to a reaction mixture containing ^{125}I-toxin with or without unlabeled toxin. The samples were mixed and incubated for

Test pulses to -10 mV were applied at 0.1 Hz rate from a E_H of -70mV. The inactivation rates were determined botn by measuring the time at which the peak current (I_{peak}) had decayed to 1/e, or by taking the larger time constant (t_1) obtained from fitting the inactivation with one or two exponentials terms, because it is known that the number of exponential terms vary from oocyte to oocyte (Trimmer *et al.*, 1989; Zhuo *et al.*, 1991; Chen *et al.*, 1992).

RESULTS

Lethal dose comparison of soluble venoms

Lethality tests conducted with these venoms indicated that the LD_{50} value for *C. i. infamatus* is 1.27 mg/Kg of mouse weight (Dehesa-Dávila and Possani, 1994a, 1994b). This value is about 70% less than that (3.30 mg/Kg) reported by Alagón *et al.* (1988) for *C. l. limpidus*, and about the double of that (0.69 mg/Kg) determined for the sub-species *C. l. tecomanus* (Possani *et al.*, 1980). *C. noxius* venom has the lowest LD_{50} value in mice: only 0.26 mg/Kg (Dent *el al.*, 1980), while that of *C. sculpturatus* is 1.12 mg/Kg (Stahnke, 1963), and that of *C. santa maria* is 0.39 mg/Kg mouse (Zlotkin *et al.*, 1971). The variations shown in the LD_{50} values of these different species of scorpions are comparable to those reported between different strains of mice used for lethality determination with venom from one specie *C.l. limpidus* (Alagón et al., 1988).

Purification procedures

Separation of soluble venom from the three species under study, gave essentially the same results as previously reported (for *C. noxius*, Possani *et al.*, 1981, for *C.l limpidus*, Alagón *et al.*, 1988 and for *C.i infamatus*, Dehesa-Dávila *et al.*, 1994b), with slight variations, mainly due to the amount of material applied to the chromatographic columns.
For comparative purposes, here are included the results obtained for Cii and Cl1, the two least studied species. The profile of Sephadex G-50 (fine) separation of 29.5 mg soluble venom from *C.i. infamatus* is shown in Fig.1a. In Fig.1b are the corresponding results obtained with 28.1 mg of *C. l. limpidus* venom. At least six components are evident in this figure for both venoms, with a clear difference on fractions II and III. These fractions in Cii venom are not well separated (see also Dehesa-Dávila et al., 1994b). If more *Cl1* scorpion venom is applied to the column, the separation of fractions II and III is very poor (Alagón et al., 1988). However, for both venoms only fraction II was lethal to mice, in our experimental conditions, and this amounts to approximately 42% for Cl1 and about 44% for Cii venom (Table 1). Overall chromatographic recovery was 96% for

Cii and 97% for Cl1. Fig. 2 and 3 shows further separation of the toxic fractions II using a combination of CM-32 ion exchange chromatographic columns and HPLC. In Fig. 2a are the results of separating Cii fraction II and in Fig.2b the separation of fraction II from Cl1. The final recoveries were 82% and 99%, respectively (Table 1). Despite the fact that these profiles are different, they show at least 13 distinct sub-fractions, from which the last five (most basic ones) are all toxic to mice, as indicated by T in the Figs. The most abundant among the mammalian toxins are fractions II.11 for Cii and II.9 for Cl1. Fraction II.11 of Cii (Fig.2a) corresponds to 18.3% of protein recovered in this chromatographic step, and fraction II.9 of Cl1 (Fig.2b) is 13.7% of the material recovered. Component II.13 from Cl1 (Fig. 2b) was obtained by washing the column with 1 M NaCl and was shown to be toxic only to crustaceans (sweet water shrimps), confirming data previously reported by Alagon *et al* (1988). Toxic component II.11 from Cii was further separated by HPLC giving three main protein peaks, the most important (number 2) eluting at 32.6 min (Fig. 3a). This latter peak was shown to be toxic to mice, gave a single protein band on PAGE, using the Reisfeld technique (Reisfeld et al., 1962; data not shown), and was sequenced and used for electrophysiological experiments. Similarly, toxic component II-9 from Cl1 was applied to a CMC-cellulose column pH 6.0 (Fig. 3b) originating 6 sub-fractions, from which number 4 was toxic to mice and was further separated by HPLC (Fig.3c). The most important component was number 3, with a retention time 41.2 min. It was re-applied onto an analytical C18 reverse-phase column (Vydac) and the main component (inset Fig.3c) was toxic and used for additional characterization.

Sequence determination

An aliquot (1.0 nmol) of each of the purified toxins was loaded in a microsequencer (Prosequencer-Millipore) and directly sequenced.
The toxin from Cii turned out to be the same as toxin 1, recently reported by our group (Dehesa-Davila et al., 1994, Toxicon), while the toxin from Cl1 was different from toxin Cl1-1 previously described by Ramirez et al., 1994, and thus was called toxin Cl1 2. To complete the primary structure determination of both peptides, 10 nmoles of Cii 1 and 7 nmoles of Cl1 2 were reduced and alkylated with iodoacetic acid, and digested with protease V8 from *Staphylococus aureus* and trypsin in order to obtain fragments of the toxins. Typical HPLC separations of digested toxins are shown in Fig.4a for Cl1 2 and Fig.4b for Cii 1. The overlapping segments that complete the primary structure are shown in Fig.5. The first 48 amino acid residues of toxin Cii 1 were determined by direct Edman degradation. Two fragments (V8-1 and V8-2) were separated after protease V8 cleavage and corresponded to residues at positions 29 to 53 and 54 to 66, respectively. The overlapping positions were obtained by

sequencing a tryptic peptide (T, in Fig.5) that corresponds to residues numbers 36 to 57. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and amino acid analysis of toxin Cii 1 (data not shown) indicated a mol. wt. in the order of 7600, compatible with the presence of 66 amino acid, as determined by sequencing data.
For toxin Cl1 2 the first 34 amino acids were identified by direct Edman degradation. Two segments were positioned after sequencing V8 cleaved peptides (V8-1 and V8-2 in Fig.5) of positions 29 to 53 and 54 to 65, with trace amounts of Asn at position 66. The overlapping peptide, was a tryptic peptide (T), which provided the amino acid sequences from position 36 to 61. The last amino acid residue (Asn) was confirmed by the amino acid analysis of the C-terminal peptide (V8-2), which gave the following composition: 2 Lys, 1 Asp, 1 Thr, 1 Glu, 2 Pro, 1 Ala, 1 Leu, 2 Val, compatible with the sequence found. We have surmised that the last amino acid is Asn, because acid hydrolysis for amino acid analysis converts Asn into Asp. Carboxymethyl-cysteine was not integrated by the analyzer, and Trp is usually degraded by the HCl hydrolysis, however both residues were clearly shown in the sequence. For Cl1 2 also the mol. wt. found is in the order of 7600 (electrophoresis data not shown).

Binding and displacement experiments
In order to study the specificity and the binding properties of these newly purified and characterized peptides, the well known toxin (toxin 2 from *C. noxius*) was used as a model. Sitges *et al.* (1987) using brain synaptosomes have shown that component II-9.2.2 of *C. noxius* (now simply called toxin 2, after Zamudio *et al.*, 1992), was specific for Na^+ channels, and behaved as a ß-scorpion toxin. Ramirez *et al.* (1994) radio-labeled this peptide and determined its binding properties to the membranes of the same rat brain preparation.
In Fig.6 we show the results of binding and displacement experiments conducted with *C. noxius* ^{125}I-toxin 2 and toxin Cii 1 and Cl1 2. The three toxins displace in an almost overlapping manner the binding of each other to rat brain synaptosome membranes.

Toxin assays on heterologously expressed µI-2 Na^+ channels
The purified scorpion toxins were functionally assayed on the µI-2 rat skeletal muscle Na^+ channel clone (Trimmer *et al.*, 1989), heterologously expressed in *Xenopus* oocytes. Fig. 7 shows typical Na^+ currents elicited by a -10 mV test pulse depolarization from µI-2 injected oocytes held at -70 mV E_H. Control (open symbols) and toxin-treated (closed symbols) current records are superimposed. All three toxins, Cii toxin 1 (panel A), Cl1 toxin 2 (panel B) and Toxin 2 from *C. noxius* venom (panel C) reduced the peak amplitude of the Na^+ currents in a reversible manner, with no efect on the channel inactivation, as measured by both the slow inactivation rate (t_1), or by the time at wich I_{peak} had decayed to 1/e

(Table 2). Both Cii and Cl1 have similar IC_{50} values, in the micromolar concentration range, as shown in table 2. In this system toxin 2 is only sligthly more potent than the other two toxins, and these results support the conclusion that all three toxins belong to the ß-type of Na^+ channel scorpion toxins.

DISCUSSION

C. infamatus infamatus and *C. limpidus limpidus* are morphologicaly related species, separated by a natural barrier (high mountains of Sierra Madre Occidental). Both species are responsible for an important number of human accidents in three states of Mexico: Guanajuato, Morelos and Guerrero (Dehesa-Davila and Possani, 1994a, 1994b). However, as mentioned in the introductory section much less is known about the structure and function of the toxic peptides present in *C. infamatus infamatus* venom, contrary to the species *C. noxius* from Nayarit state, which were extensively studied (Dent *et al.*, 1980, Carbone *et al*, 1982, 1987; Possani, 1984; Zamudio *et al*, 1992; Becerril *et al*, 1993, Valdivia *et al.*, 1994). Chromatographic separation of these three venoms by Sephadex G-50 (Fig.1) show similar patterns. They all have an important fraction (mol wt. range 4,000 to 12,000), numbered II in the figures that contains the toxic peptides to mice. Chromatographic application of fraction II on cation exchange resins retain the basic peptides, which are eluted with a salt gradient. The last five components (Fig.2) are all toxic to mammals, except component 13 of Cl1 which is toxic to crustaceans. Additional separation of the most important toxic sub-fractions: II-9 and II-11 respectively for Cl1 and Cii, and II-9 for C. noxius (ref. Zamudio, 1992), by ion exchange column or by HPLC (Fig.2 and 3), permits to obtain in homogeneous form the major toxic peptides of each one of these venoms. Automatic Edman degradation of native, RC-toxins and peptides obtained after enzymatic cleavage (Fig.4) allowed us to determine the full primary sequences of the first two unknown toxins (Fig.5). The similarity between these two toxins is striking, only two amino acid are changed: Tyr and Phe at position 17 and Thr and Asn at position 49, respectively for Cii 1 and Cl1 2.

Comparative analysis of the sequences obtained with other known toxins (Fig.8) shows that the Cii 1 corresponds to toxin 1 of the same venom, for which the N-terminal part of the molecule was already known (Dehesa-Davila and Possani, 1994b). In this manner this is the first toxin from *C.i.infamatus* species, and the only one thus far, to have the full primary structure determined. At this moment, for the species *C.l.limpidus* there are several different toxins known. Four are toxins specific for Na^+ channels (toxin Cl1 2, this communication), toxin 1 (Ramírez et al., 1994), and toxin II-6 and II-9 (Alagon et al., 1988). Two other short-chain toxins specific for K^+ channel (Martin *et al.*, 1994) and one specific for crustaceans (Lebreton *et al.*, 1994) are also known.

Comparative analysis of Fig.8 clearly shows a high degree of similarity (83% and over) among the mammalian toxins of *C.i.infamatus*, *C.l.limpidus*, *C.l.tecomanus*, *C. noxius*, and *C. suffusus suffusus*. The cricket toxins (variant v1 to v3) of *C. sculpturatus* and toxin 1 from *C. noxius* are less identical (ranging from 56 to 59% identity). Recently, unpublished observations of our group with toxin 1 from *C. noxius* (previously called component II-14, Possani *et al.*, 1985) showed that this peptide is rather toxic to crustaceans, with little if any, toxicity to mammals. Thus, comparative analysis of primary structures of toxic peptides from related scorpion species indicates possible specificity of action. Concerning the function of toxins Cii 1, Cl1 2 and Cn 2, two approaches were used. The first was to assay by binding and displacement experiments the affinities of these toxins to rat brain synaptosome membranes (Fig.6). Since toxin Cn 2 is a well known ß-scorpion toxin (Ramírez *et al.*, 1994, Gurrola *et al.*, 1994, Valdivia *et al.*, 1994) that modifies the peak permeability of Na^+ channels, it was radiolabeled and used for binding experiments. The two novel toxins (Cii 1 and Cl1 2) displaced competitively the binding of Cn 2 in an almost identical manner as native (unlabeled) Cn 2 (Fig.6). Thus, they must be compiting for the same sub-types of brain Na^+ channels in a similar fashion as the ß-scorpion toxins.

In order to substantiate this conclusion in a more conclusive manner we studied these three peptides, for the first time, in an *in vitro* assay using frog oocyte expression of a specific messanger that codes for the μI2 Na^+ channel of skeletal muscle of rat. This second approach (Fig.7 and table 2) confirms indeed that these toxins are Na^+ channel specific and are all able to block the skeletal muscle μI-2 Na^+ channel in a similar fashion.

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FIGURE LEGENDS

Fig. 1: Gel filtration on Sephadex G-50

A. Soluble venom from *C.i.infamatus* (29.5 mg by absorbance at 280 nm, in 2 ml) was applied into a Sephadex G-50-fine column (0.9 x 200 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7. The flow rate was 25 ml/hr and fractions of 2.5 ml volume per tube were collected and pooled according to the absorbance at 280 nm, as shown by the horizontal bars. Only fraction II was toxic to mammals.

B. Soluble venom from *C.l.limpidus* (28.1 mg by absorbance at 280 nm, in 2 ml) was applied and run into the same column and conditions as A.

L means lethal fraction.

Fig. 2: Ion exchange separation of fraction II

A. Fraction II from *C.i.infamatus* (43.8 mg pooled from independent applications as those of Fig.1a) was applied to a CM-cellulose column (0.9 cm x 35 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 30 ml/hr. A linear salt gradient from 0 M NaCl (250 ml) to 0.5 M NaCl (250 ml) in the equilibration buffer resolved 13 components.

B. Fraction II (92.4 mg) from *C.l.limpidus* (Fig.1b) was applied and eluted into the same column and conditions as A, separating 12 fractions. Washing (W) the column with 1M NaCl resolved 1 additional component.

L and G means, respectively, loading the sample and starting point of the gradient. T denotes toxic or lethal fraction. Recoveries and toxicity tests are shown in Table 1.

Fig. 3: Final purification of toxin Cii 1 and Cll 2.

A. Toxic fraction II.11 (100 µg) from *C.i.infamatus* (Fig.2a) was fractionated by HPLC in a C18 analytical column (Vydac), using a linear gradient of solution A (0.12% TFA in water) to 60% solution B (0.1% TFA in acetonitrile) during 1 hr. Component number 2 was shown to be homogeneous as demostrated by the gel electrophoresis and amino acid sequence.

B. Toxic fraction II.9 (25.3 mg) from *C.l.limpidus* (Fig. 2) was applied to a CM-cellulose column (0.9 x 30 cm) equilibrated and run in 50 mM potassium phosphate buffer, pH 6.0 at flow rate of 30 ml/hr. A linear gradient of salt from 0 M NaCl (250 ml) to 0.4 M NaCl (250 ml) in the same buffer resolved 6 components.
Fraction 4 was the only toxic (T) to mice.
L and G are respectively, loading the sample and starting point

of the gradient.

C. Toxic fraction II.9.4 (100 µg) from *C.l.limpidus* was separated by HPLC in a C4 column (Vydac) with a linear gradient identical to that of A. Component 3 was toxic and was further separated into an analytical C18 reverse-phase column by HPLC (inset), in the same conditions as C. Component 2 was homogenous as demostrated by gel electrophoresis and amino acid sequence.

Fig. 4: HPLC separation of cleaved peptides

a. HPLC separation of RC-toxin Cll 2 (50 µg) after hydrolysis with protease V8 from *Staphylococcus aureus*. The four peptides labeled 1-4 corresponded to sequences 3 to 15 (GYLVNHSTGCKYE), 54-65 (QAVVWPLPKKTC), 16-28 (CFKLGDNDYCLRE), and 29-53 (CKQQYGKGAGGYCYAFGCWCNHLYE), respectively (see Fig.5).

b. HPLC separation of tryptic digestion of RC-toxin Cii 1 in the same conditions as a. Peptide with the star (eluting approximately at 44 min) corresponds to the the overlapping sequence of positions 36 to 57 (GAGGYCYAFGCWCTHLYEQAVV) of Fig.5.

Fig. 5: Primary structure of toxin Cii 1 and Cll 2

Cii 1. The overlapping amino acid sequences of toxin 1 from *C.i.infamatus* is shown on the upper panel.
Residues at positions 1 to 48 were determined by direct Edman degradation of RC-toxin (labeled -D->). Peptides labeled with (.v8-1.>) and (.v8-2.>) were obtained by enzymatic cleavage of RC-toxin with protease V8. The first corresponds to positions 29 to 53 and the second to 54 to 66. The overlapping peptide (.-T.->) was obtained after trypsin cleavage and spans from residues 36 to 57.

Cll 2. The amino acid sequence of toxin 2 from *C.l.limpidus* is shown on the lower panel of the figure.
Residues at positions 1 to 34 were directly determined from RC-toxin (labeled -D->), while peptides v8-1 and v8-2 corresponds to the sequences from 29 to 53 and 54 to 65, respectively, as in Cii 1. The overlapping peptide (.-T.->) was a tryptic digestion of TC-toxin and provided the sequence from positions 36 to 61. The last residue: Asn was confirmed by amino acid analysis of the C-terminal peptide (labeled a).

Fig. 6: Displacement of ^{125}I-Cn2 binding to rat brain synaptosome membranes

Displacement experiments were conducted using rat brain synaptosome membranes (80 µg/500 µl per assay) incubated with 10 nM of ^{125}I-toxin 2 from *C. noxius* (Cn2), at room temperature for

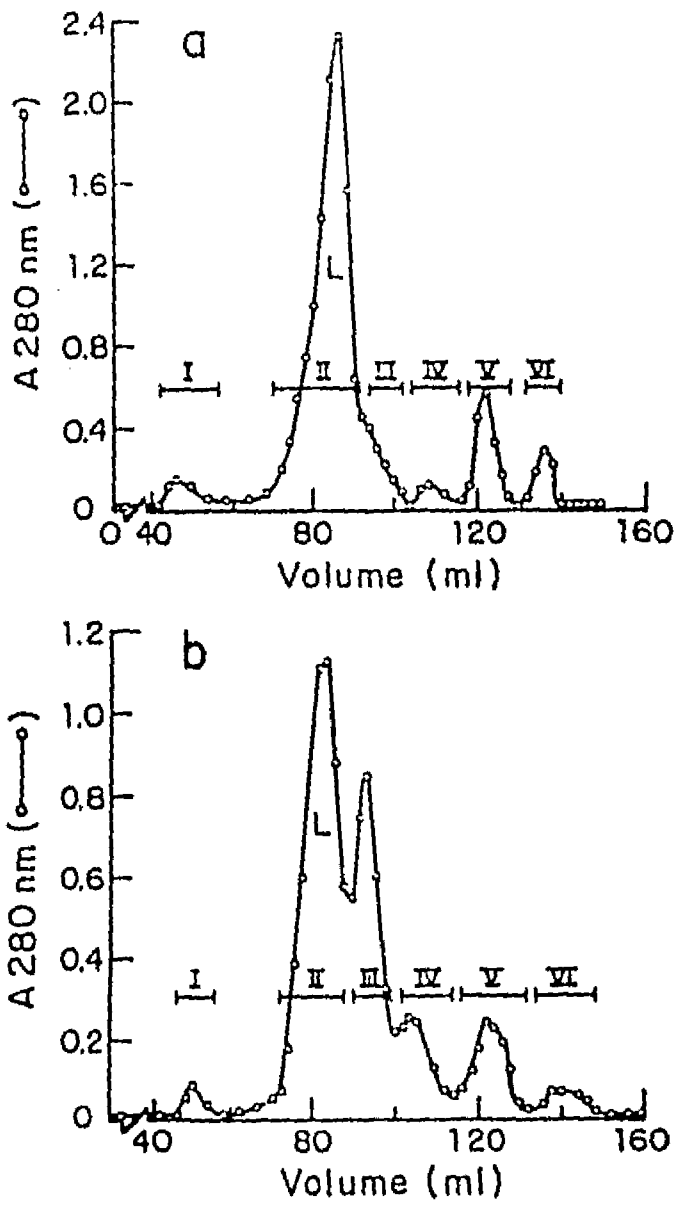
60 min, in the presence of increasing concentrations of native Cn2 (filled circles) or toxin 2 from Cl1 (open circles) or toxin 1 from Cii (triangles), filtered and counted, as described in Materials and Methods. The 100% binding was obtained without competing toxin. Values are mean of triplicates.

Fig. 7: Effect of scorpion toxins on the rat μI-2 skeletal muscle channel.

μI-2 cRNA injected *Xenopus* oocytes whole-cell Na^+ currents were elicited by depolarizations to -10 mV from a holding potential of -70 mV. Control (open symbols) and toxin-treated (closed symbols) current records are superimposed. The toxin concentrations used were: 1μM for Cii toxin 1 (A, closed circles) and Cl1 toxin 2 (B, closed squares) and 0.3 μM for Cn toxin2 (C, closed diamonds).

Fig. 8: Comparison of primary structure of toxins from Mexican scorpions

The one letter code for amino acids was used to compare the primary structure of toxins: Cii 1 and Cl1 2, are from this work; Cl1 1 (*C. limpidus limpidus* 1) from Ramírez et al., (1994); Clt 1 (*C. limpidus tecomanus* 1) from Martin et al., (1988); Cn 1 (*C. noxius* 1) from Possani et al., (1985); Cn 2 and 3 (*C. noxius* 2 and 3) from Zamudio et al., (1992); Cn 4 (*C. noxius* 4) from Vázquez et al., (1993); Cas II (*C. suffusus suffusus* II) from Rochat et al., (1979); CsE I, V1 to V3 (*C. sculpturatus* Ewing I, V1 to V3) from Babin et al., (1975); Cl1 c1 (*C. limpidus limpidus* crustacean toxin 1) from Lebreton *et al.*, 1994. Percentages of identity are listed on the right of the figure. The first number on top of the sequences corresponds to the amino acid position in the sequence. A couple of gaps (-) were introduced in the sequence in order to align the cysteines (in bold). The last sequence, labeled common indicate the positions of identical amino acids in all 13 structures compared (27 out of 67 positions) and also are in bold, separated by x (non identical residues).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2.0
1.6
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0.4
0
A 280 nm
L
I
II
III
IV
V
VI
0 40 80 120 160
Volume (ml)
b
1.2
1.0
0.8
0.6
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A 280 nm
L
I
II
III
IV
V
VI
40 80 120 160
Volume (ml)

FIG. 1

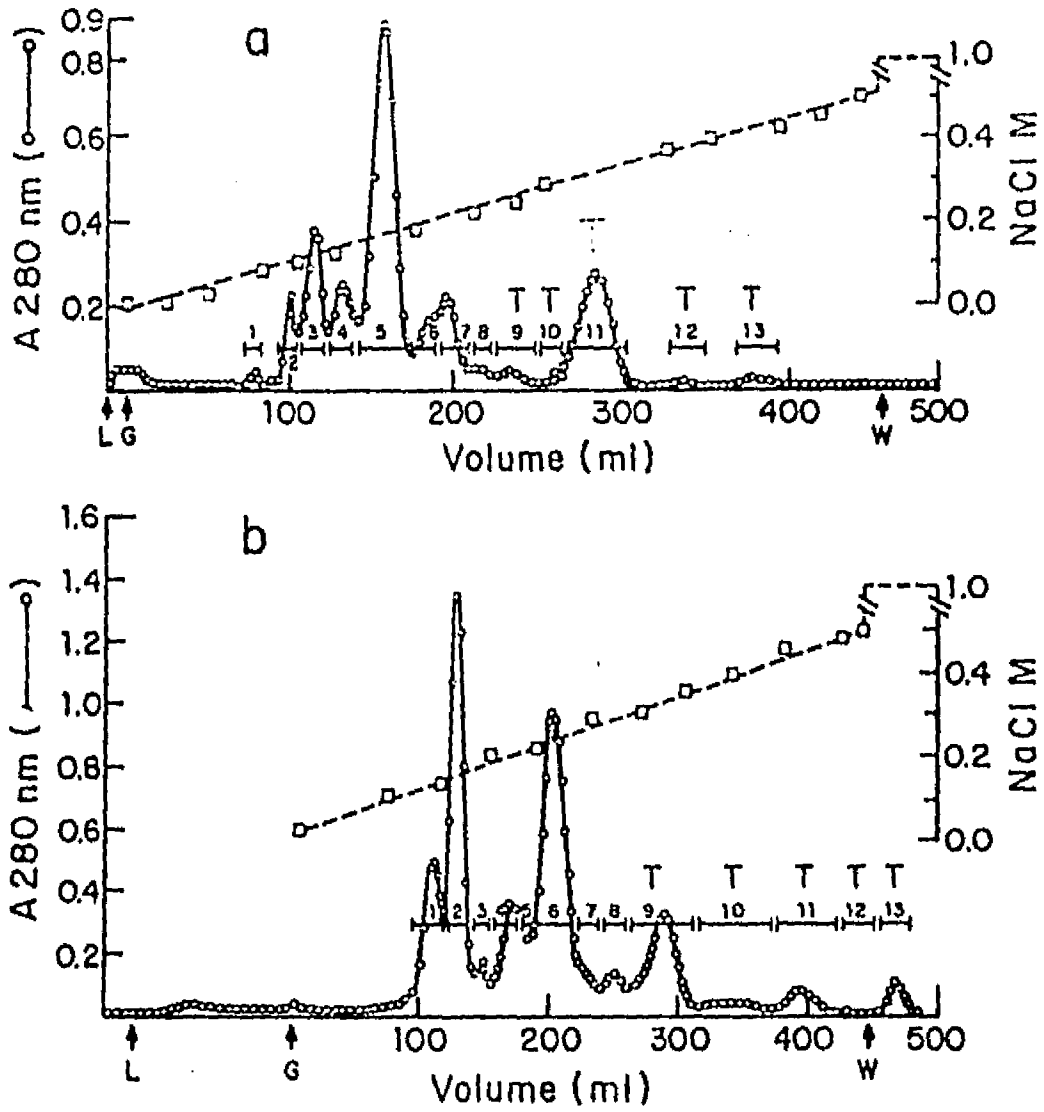
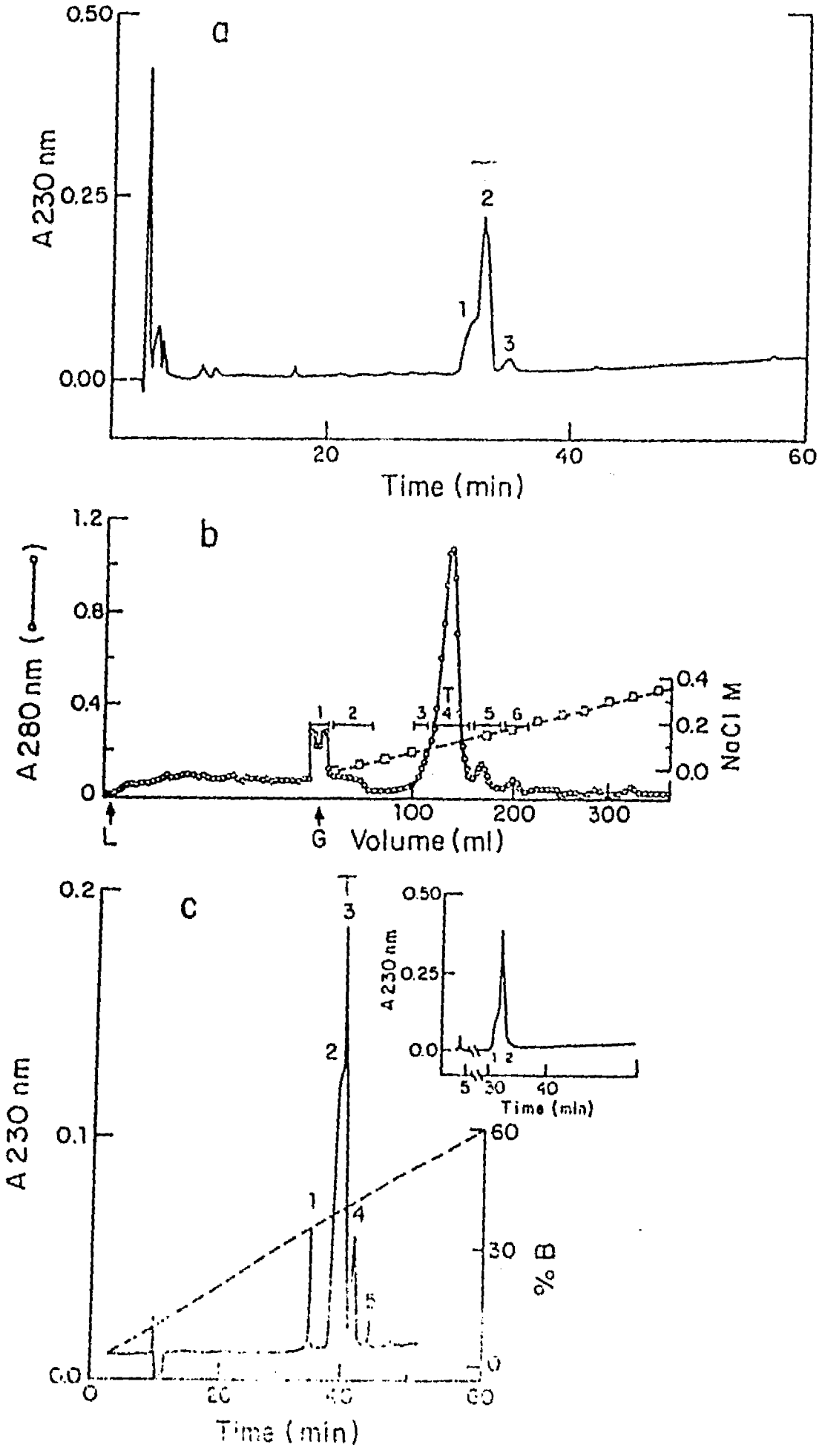


FIG. 2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0.00
A 230 nm
1
2
3
20
40
60
Time (min)
b
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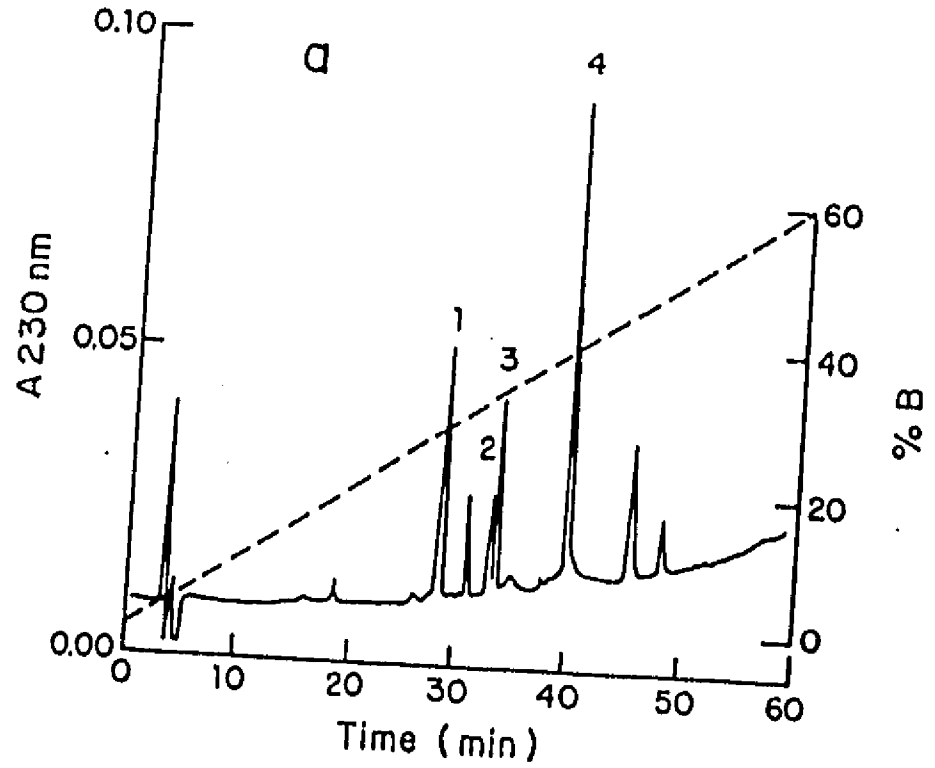
0.8
0.4
0
A 280 nm
0.4
0.2
0.0
NaCl M
100
200
300
L
G
Volume (ml)
C
0.2
0.1
0.0
A 230 nm
0
20
40
60
Time (min)
30
% B
0.50
0.25
0.0
A 230 nm
5
30
40
Time (min)

FIG. 3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A 230 nm
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% B
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10
20
30
40
50
60
Time (min)

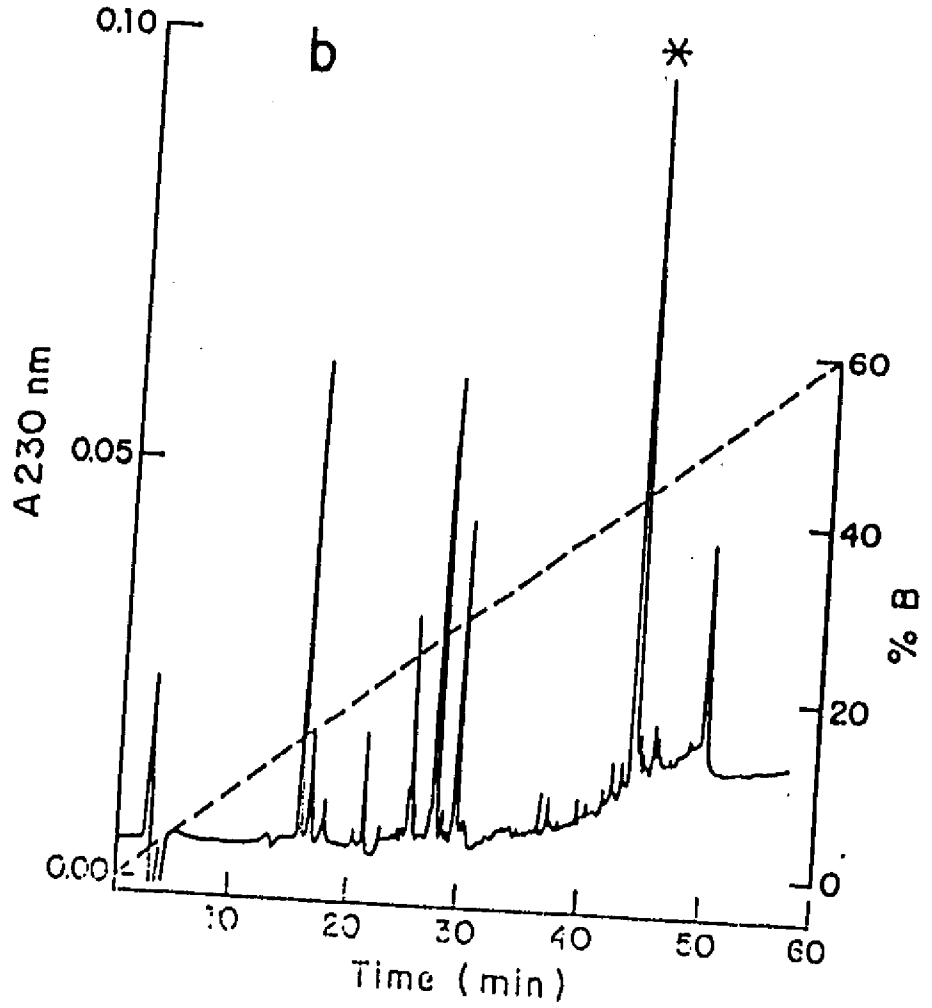
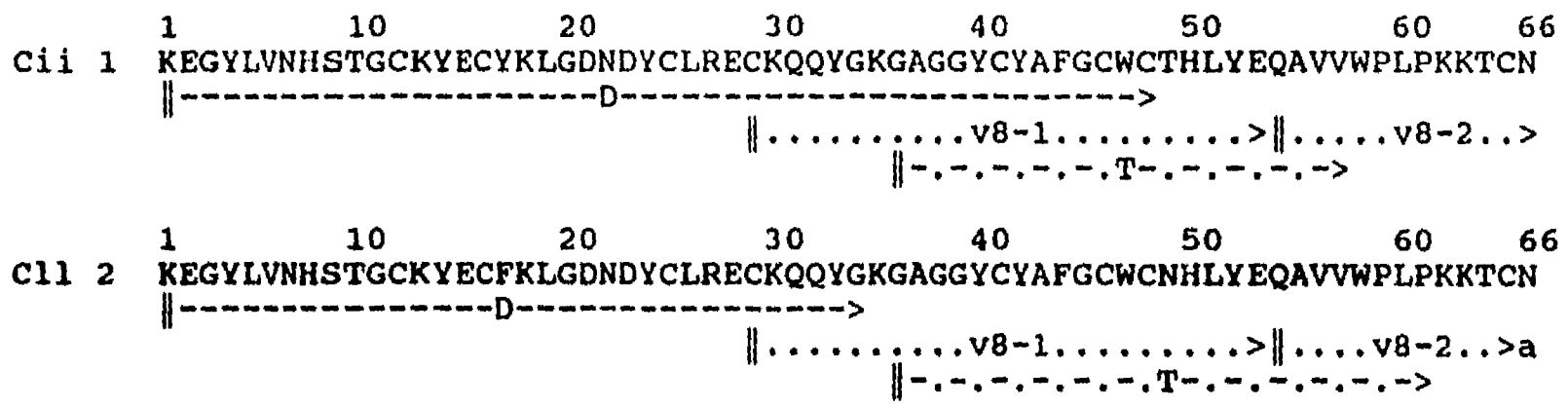

b
0.10
0.05
0.00
A 230 nm
*
60
40
20
0
% B
10
20
30
40
50
60
Time (min)

FIG.4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‖----------D----------->
‖.....v8-1.....>‖.....v8-2..>
‖-.-.-.-.-.-.T-.-.-.-.-.->
1 10 20 30 40 50 60 66
C11 2 KEGYLVNHSTGCKYECFKLGDNDYCLRECKQQYGKGAGGYCYAFGCWCNHLYEQAVVWPLPKKTCN
‖----------D----------->
‖.....v8-1.....>‖....v8-2..>a
‖-.-.-.-.-.-.-.T-.-.-.-.-.->
Fig. 5

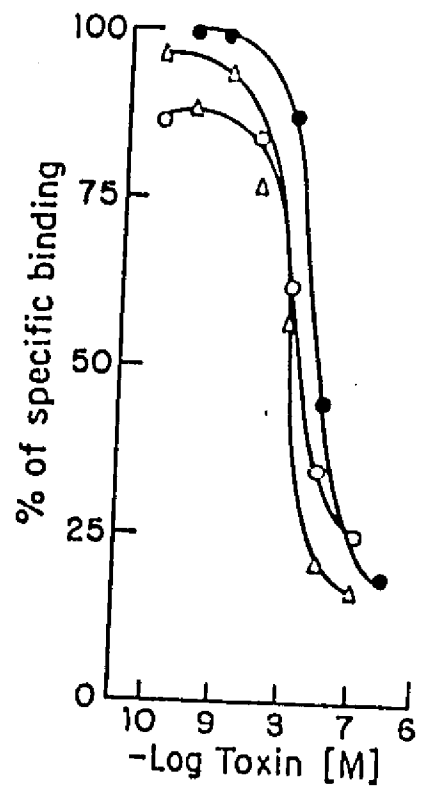

FIG.6

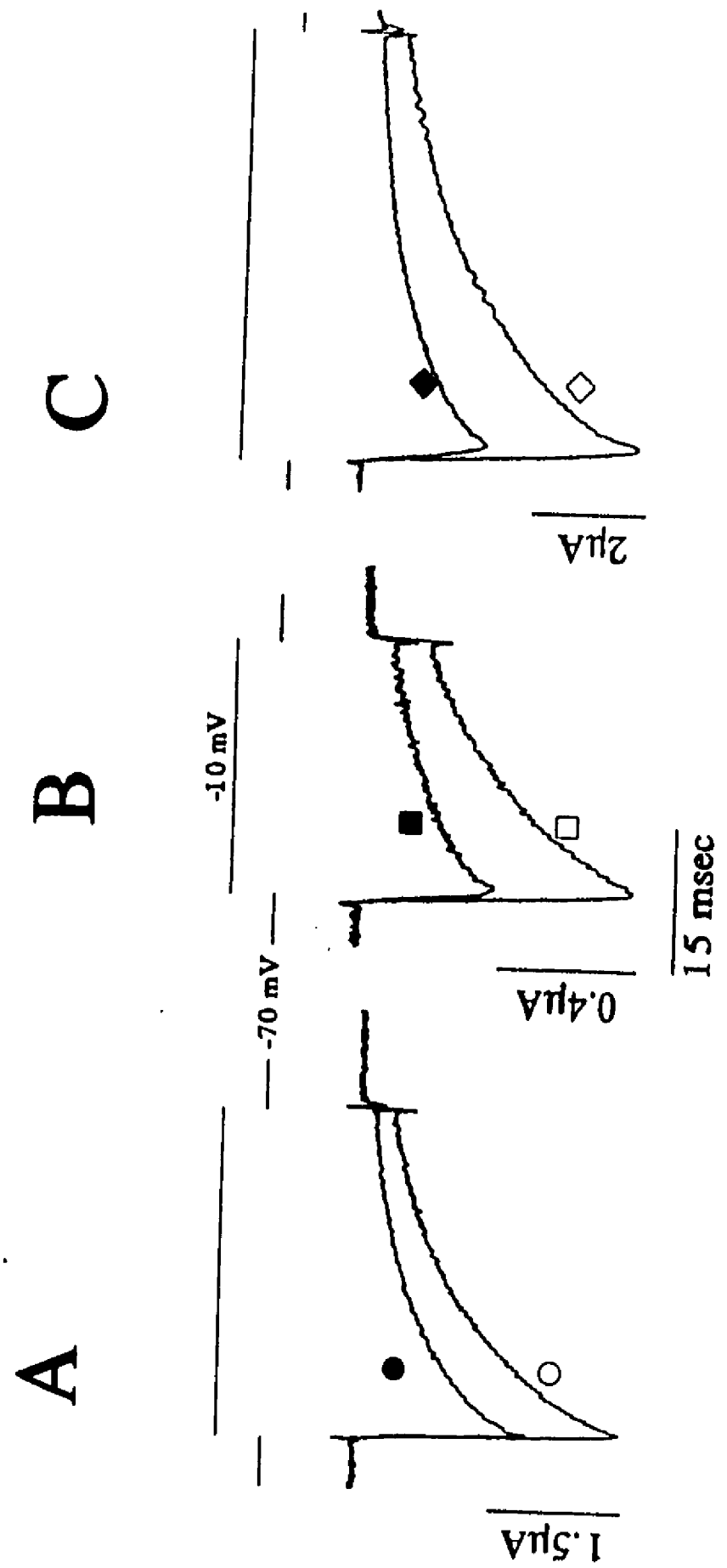

Fig. 7

```
          1        10        20        30        40        50        60    66  %
Cii 1     KEGYLVNHSTGCKYECYKLGDNDYCLRECKQQYGKGAGGYCYAFGCWCTHLYEQAVVWPLPKKTCN 100
Cll 2     KEGYLVNHSTGCKYECFKLGDNDYCLRECKQQYGKGAGGYCYAFGCWCNHLYEQAVVWPLPKKTCN  97
Cll 1     KEGYIVNLSTGCKYECYKLGDNDYCLRECKQQYGKGAGGYCYAFGCWCTHLYEQAVVWPLPKKTCT  95
Clt 1     KEGYLVNHSTGCKYECFKLGDNDYCLRECRQQYGKGAGGYCYAFGCWCTHLYEQAVVWPLPNKTCS  94
Cn  4     KEGYLVNSYTGCKYECFKLGDNDYCLRECKQQYGKGAGGYCYAFGCWCTHLYEQAVVWPLKNKTCN  92
Css II    KEGYLVSKSTGCKYECLKLGDNDYCLRECKQQYGKSSGGYCYAFACWCTHLYEQAVVWPLPNKTCN  89
Cn  3     KEGYLVELGTGCKYECFKLGDNDYCLRECKARYGKGAGGYCYAFGCWCTQLYEQAVVWPLKNKTCR  85
Cn  2     KEGYLVDKNTGCKYECLKLGDNDYCLRECKQQGYKGAGGYCYAFACWCTHLYEQAIVWPLPNKRCS  83
CsE V2    KEGYLVNKSTGCKYGCLKLGENEGCDKECKAKNQGGSYGYCYAFACWCEGLPESTPTYPLPNK-CSS 59
CsE I     KDGYLVEK-TGCKKTCYKLGENDFCNRECKWKHIGGSYGYCYGFGCYCEGLPDSTQTWPLPNK-CT  59
CsE V3    KEGYLVKKSDGCKYGCLKLGENEGCDTECKAKNQGGSYGYCYAFACWCEGLPESTPTYPLPNKSC-  58
Cn  1     KDGYLVDA-KGCKKNCYKLGKNDYCNRECRMKHRGGSYGYCYGFGCYCEGLSDSTPTWPLTNKTC-  58
Cll c1    KEGYLVNKSTGCKYGCFWLGKNENCDKECKAKNQGGSYGYCYSFACWCEGLPESTPTYPLPNKSCS  56
CsE V1    KEGYLVKKSDGCKYDCFWLGKNEHCNTECKAKNQGGSYGYCYAFACWCEGLPESTPTYPLPNK-CS  56
Common    KxGYLVxxxxGCKxxCxxLGxNxxCxxECxxxxxxxxxGYCYxFxCxCxxLxxxxxxxPLxxKxCxx
```

Fig. 8

Table 1. Recovery and toxicity of chromatographic components

A. Sephadex G-50 column (Fig.1a and b)

Protein component	Amount in mg (A_{280} nm)* Cii	Cll	Lethality**
Venom	29.5	28.1	Lethal
F I	1.5	1.1	Nontoxic
F II	12.3	12.3	Lethal
F III	7.0	7.3	Nontoxic
F IV	1.7	2.4	Nontoxic
F V	3.5	2.9	Nontoxic
F VI	2.2	1.2	Nontoxic
Protein recovered	96%	97%	

B. CMC-32 column (Fig.2a and b)

Protein component	Amount in mg (A_{280} nm)* Cii	Cll	Lethality**
F II	43.8	92.4	
F II.1	0.6	8.2	Not tested
F II.2	1.2	16.4	Not tested
F II.3	4.4	2.8	Not tested
F II.4	3.1	7.1	Not tested
F II.5	13.0	3.1	Non toxic
F II.6	1.9	22.9	Non toxic
F II.7	3.0	5.0	Non toxic
F II.8	0.7	3.7	Non toxic
F II.9	0.8	12.5	Toxic
F II:10	0.2	3.5	Toxic
F II.11	6.6	3.4	Toxic
F II.12	0.1	1.2	Toxic
F II.13	0.4	1.7	Toxic
Protein recovered	82%	99%	

*Assuming one Absorbance unit at 280 nm equals to 1 mg/ml protein
**Lethality defined in the section of Materials and Methods

Table 2. Time constants of inactivation and IC_{50} values in control and toxin treated oocytes*

	$0.37^{*}I_{peak}$ (msec)	t_1 (msec)	IC_{50} (μM)
Control	14.5 ± 2.5 (10)	12.5 ± 1.3 (10)	
Cii toxin 1	12.9 ± 4.2 (6)	13.0 ± 1.8 (6)	1.7 ± 0.4 (5)
Cll toxin 2	16.2 ± 2.2 (5)	12.8 ± 4.0 (5)	1.2 ± 0.8 (5)
Cn toxin 2	16.7 ± 1.1 (3)	13.9 ± 1.7 (3)	0.5 ± 0.2 (3)

*Values are mean ± s.d. and number of experiments are enclosed by parenthesis

IV SECRECIÓN EXÓCRINA Y MICROSCOPÍA

Los ensayos de secreción se llevaron a cabo en ratones de 20 g de peso corporal *in vivo*. Fueron retados con veneno total de *C. limpidus limpidus* a dosis de 20 μg aplicados por vía intraperitoneal. El método que se utilizó para cuantificar la enzima amilasa en suero fue el de Bernfeld (1955) que consiste brevemente en expresar la actividad de amilasa en términos de liberación de maltosa en 3 minutos. Antes del reto se les extrajo 100 μl de sangre como control. Los animales una vez que fueron retados se les extrajo sangre cada hora hasta completar 4 hrs. Después fueron sacrificados por dislocación cervical y el páncreas removido para estudio de microscopia. El páncreas fue fijado con medio de Karnovsky.

En los resultados de secreción no hubo respuesta siendo las cuantificaciones iguales a los controles por ello no se muestran estos resultados. De los estudios de microscopía de luz se muestran dos figuras. En la Fig A está el control donde se observan acinos pancreáticos con arquitectura conservada, se destaca abundante cantidad de gránulos de zimógeno intracitoplasmáticos teñidos con azul de toluidina, dispuestos en acúmulos en forma difusa sin alteraciones ductales y capilares . En la Fig. B se observa la misma arquitectura conservada de los acinos pancreáticos con alteraciones en la coloración del citoplasma y marcada disminución de los gránulos de zimógeno dispuestos en forma de predominio ductal. En la periferia se observan focos de esteatosis necrótica.

De estos experimentos se concluye que si bien en la microscopía de luz se observan algunos cambios cualitativos en los de secreción no los hay. Los venenos de los alacranes del género *Centruroides* parecen no afectar de forma importante al páncreas. Sin embargo, el trabajo llevado a cabo por el Dr. Fletcher *et al.*, (1992) y el Dr. Possani *et al.*, (1991) donde se muestra que el veneno de *Tityus serrulatus* y toxinas purificadas del

mismo sí tienen un efecto sobre la secreción exócrina del páncreas con alteraciones histopatológicas compatibles con una pancreatitis aguda estimulan el continuar con estos experimentos para elucidar la relación existente entre toxinas purificadas y los mecanismos de secreción.

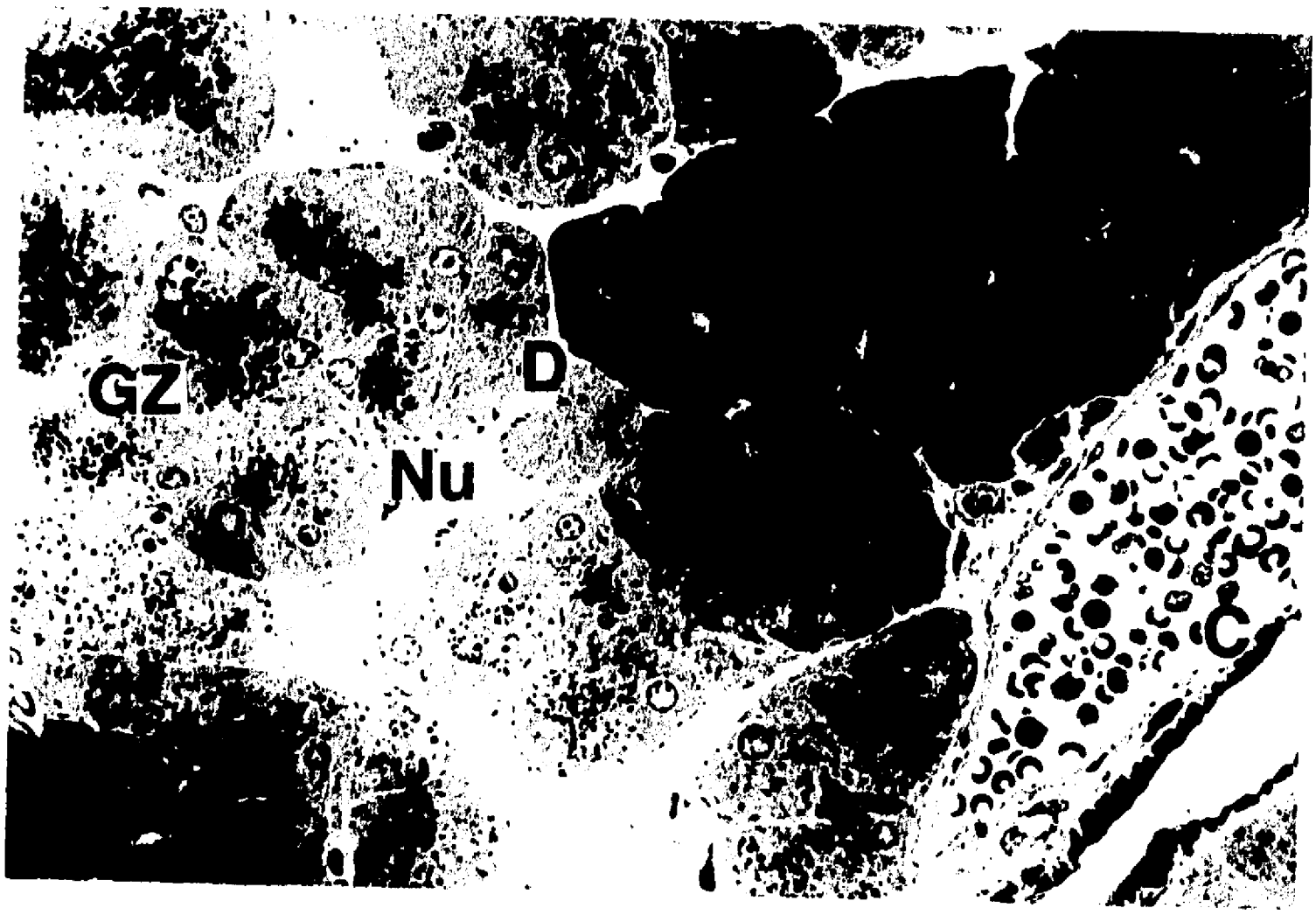

Fig. A

(Control) Microscopía de luz que muestra un conjunto de acinos pancreáticos con arquitectura celular conservada. Los gránulos de zimógeno (GZ) están dispuestos en acúmulos difusos en el citoplasma. Los ductos (D) no presentan alteración alguna al igual que los capilares (C). El núcleo (Nu) se encuentra de forma y situación normal. 20 x 100 aumentos.

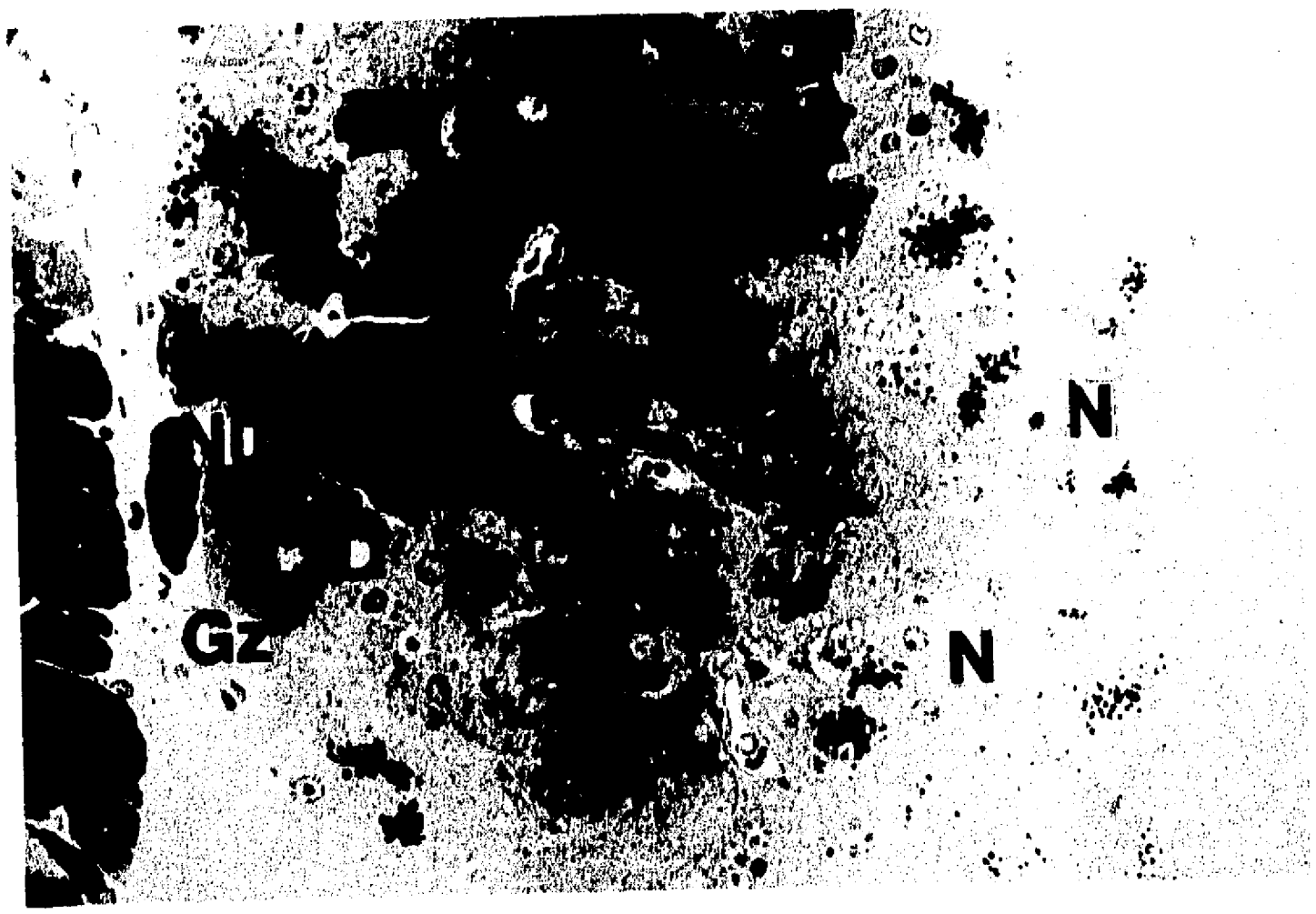

Fig. B

Acinos pancreáticos con arquitectura celular conservada, alteraciones en la coloración del citoplasma con marcada disminución de gránulos de zimógeno (GZ) orientados hacia la luz de los ductos (D) ó en sus porciones periductales. Los núcleos no presentan alteraciones. Se observa necrosis (N) grasa en la periferia del conjunto acinar. 10 x 100 aumentos.

V DISCUSION Y CONCLUSIONES

Tanto el *C. infamatus infamatus* como el *C. limpidus limpidus* son las dos especies de alacranes responsables de un importante número de envenenamientos en los estados de Guanajuato, Guerrero y Michoacán (ver anexo 2 y 3). La separación cromatográfica del veneno de *C. infamatus* y de *C. limpidus* muestra patrones similares desde su fraccionamiento en resina de Sephadex G-50 fino con una fracción importante (denominada fracción II en la cromatografía con Sephadex) la cual contiene los polipéptidos tóxicos a mamíferos (ratón). La fracción II al ser recromatografiada en resina de intercambio iónico (CMC-32) retiene los péptidos básicos los cuales son eluidos con el gradiente salino. Los últimos cinco componentes son tóxicos a mamíferos, excepto el componente número 13 del *C. limpidus* el cual es tóxico a crustáceos. Purificaciones subsecuentes de los principales componentes (II.9 de *C. limpidus* y II.11 de *C. infamatus*) por recromatografías en resina de intercambio iónico ó por cromatografía líquida de alta presión (CLAP) nos permitió obtener en forma homogénea un péptido de cada uno de estos venenos (ver fig. 2, III). La estructura primaria completa de estos dos péptidos fue obtenida por medio de la degradación automática de Edman primero, posteriormente se llevó a cabo una reducción y carboximetilación de la toxina nativa y finalmente se realizó una digestión enzimática (ver fig. 3 y 4, III). La similitud de secuencia entre estos dos péptidos, denominados toxina 1 de *C. infamatus* y toxina 2 de *C. limpidus* es extraordinaria, unicamente hay dos aminoácidos diferentes, uno es una tirosina por fenilalanina en la posición 17 y una treonina por asparagina en la posición 49 (ver figura 8, III) para Cii 1 y Cii 2, respectivamente.

En conclusión, presentamos el aislamiento y purificación de dos nuevas toxinas del veneno de alacranes mexicanos. Su secuencia de aminoácidos tiene una alta homología entre ambas siendo del 98%. De la misma manera estas dos toxinas tienen una homología con otras toxinas

aisladas del mismo género que va del 62 hasta el 98%, como sería lo esperado (ver fig. 8, III).

Jover *et al.*, (1980), describieron dos sitios diferentes para las toxinas de alacrán en sinaptosomas de cerebro de ratón. Las toxinas α representadas por alacranes del viejo mundo las cuales hacen lenta la inactivación de los canales de Na^+ y las toxinas β representadas por la toxina II de *C. suffusus suffusus* (alacranes de Norte América o del Nuevo Mundo) las cuales afectan la activación del canal de Na^+. Sin embargo en el veneno del alacrán *C. sculpturatus* hay toxinas que tienen el efecto de las toxinas β (anotado por Martin *et al*, 1988) y otras toxinas que afectan la activación del canal de Na^+. De misma forma en el veneno del alacrán *Tityus serrulatus*, del Nuevo Mundo, hay toxinas del tipo α y β (Yatani *et al.*, 1988, Kirsch *et al.*, 1989).

Para abordar el estudio de la función planteamos dos enfoques, el primero fue ensayar la afinidad de las dos toxinas utilizando experimentos de pegado y desplazamiento a membranas de sinaptosomas de cerebro de rata (ver fig. 6, III). Se seleccionó a la toxina 2 de *C. noxius* por ser una bien conocida toxina β la cual se marcó con ^{125}I. Las dos toxinas desplazaron en forma competitiva el pegado de la toxina 2 de *C. noxius* en una forma casi idéntica (ver fig. 6, III), por lo tanto ambas compiten por el mismo subtipo de canal de Na^+ en la misma forma que las toxinas β. Con el fin de apoyar este resultado utilizamos un sistema de expresión en ovocitos de rana para un mensajero específico que codifica para el canal de Na^+ de músculo esquelético de rana (ver fig. 7, III). Este experimento confirmó que estas dos toxinas modifican unicamente el pico de activación de este canal. Además, sabemos que la toxina 1 de Cii afecta el canal de Na^+ de las células nerviosas de las raíces dorsales de embrión de pollo disminuyendo el pico de activación siendo este efecto reversible después de lavado (ver anexo 1). Por lo tanto, ambas toxinas son clasificadas como toxinas de tipo β o de las llamadas del nuevo mundo.

Por demás interesante es la comparación de la toxina 1 de Cii con la toxina 1 de *C. limpidus tecomanus* puesto que fueron ensayadas en la misma preparación con concentraciones iguales. Ambas tienen un efecto relativamente pequeño que no pasa de un 25% mas su mecanismo de acción si es diferente. La toxina 1 de *C. limpidus tecomanus* hace lenta la inactivación de la corriente del canal de Na^+ en tanto la toxina 1 de Cii disminuye la activación del canal. Esto, en conjunto hace que tengamos dos toxinas con una estructura primaria muy parecida pero con función diferente que nos permitirá a futuro estudiar la función del canal de Na^+ en relación a la estructura de las toxinas.

El efecto de la toxina 1 de *C. limpidus tecomanus* es hacer lenta la inactivación del canal de Na^+ en células de las raices dorsales ganglionares de pollo en forma similar a las toxinas de los alacranes del viejo mundo, mas en células de corazón cambian el pico de permeabilidad sin modificar el curso del tiempo en la corriente durante el pulso. Ello indica que la toxina puede actuar como α o β dependiendo del tejido en que se aplica. La toxina II-10 de *C. noxius* disminuye el tamaño de la corriente en el axón gigante de calamar (Carbone *et al.*, 1982) en tanto en células de las raices ganglionares dorsales de pollo afecta principalmente el curso del tiempo de inactivación. Por ello en vez de cambios mínimos en la estructura primaria de las toxinas se debe de ver la enorme importancia de la especificidad del tejido para diferentes toxinas. Toxinas con estructura primaria similar tienen diferentes efectos en tejidos excitables de diferentes especies lo que finalmente nos lleva a la clasificación de toxinas para mamíferos, insectos y crustáceos de acuerdo al bioensayo usado durante su purificación.

Otro ejemplo es la toxina II-9 de *C. limpidus limpidus* (Alagón *et al.*, 1988) la cual es una toxina a crustáceos (en esta tesis es la toxina II-13 de *C. limpidus limpidus*) y las toxinas 1 y 2 del mismo alacrán que son toxinas a mamíferos. De esta última, la cual es objeto de la presente tesis observamos su efecto como una típica toxina β.

En resumen, podemos mencionar que el veneno de alacrán contiene una familia heterogénea de péptidos con estructura primaria variable y por ende conlleva diferentes mecanismos de acción con receptores diferentes. Por lo anterior, el descubrimiento de nuevas toxinas con diferentes estructuras y mecanismos de acción son un campo de estudio muy estimulante.

VI PERSPECTIVAS FUTURAS

Las perspectivas futuras las podemos contemplar de la siguiente forma:

1. Identificar las toxinas que afectan a los canales de K^+ de estos dos venenos tanto de *Centruroides infamatus infamatus* como de *Centruroides limpidus limpidus*.

2. Obtener por clonación los genes que codifican para toxinas que afectan tanto al canal de Na^+ como al de K^+ y tener la posibilidad de ampliar los estudios de relación estructura función con mutaciones puntuales.

3. Continuar los experimentos de secreción con toxinas purificadas en células acinares aisladas.

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ANEXO 1

Pergamon

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ISOLATION OF A TOXIN FROM *CENTRUROIDES INFAMATUS INFAMATUS* KOCH SCORPION VENOM THAT MODIFIES Na^+ PERMEABILITY ON CHICK DORSAL ROOT GANGLION CELLS

MANUEL DEHESA-DÁVILA,[1] BRIAN M. MARTIN,[2] MARIO NOBILE,[3] GIANFRANCO PRESTIPINO[3] and LOURIVAL D. POSSANI[1]*

[1]Departmento de Bioquimica, Instituto de Biotecnologia, National Autonomous University of Mexico, Av. Universidad, 2001 Apartado Postal 510-3, Cuernavaca, Morelos 62271, México; [2]National Institute of Mental Health, Section on Molecular Neurogenetics, Clinical Neuroscience Branch, Building 49 BIEE 16, Bethesda, MD 20892, U.S.A.; and [3]Istituto di Cibernetica e Biofisica, CNR, Via Dodecaneso 33, I-16146, Genova, Italy

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M. DEHESA-DÁVILA, B. M. MARTIN, M. NOBILE, G. PRESTIPINO and L. D. POSSANI. Isolation of a toxin from *Centruroides infamatus infamatus* Koch scorpion venom that modifies Na^+ permeability on chick dorsal root ganglion cells. *Toxicon* **32**, 1487–1493, 1994.—A novel toxin was isolated and characterized from the venom of the Mexican scorpion *Centruroides infamatus infamatus*. It has an apparent mol. wt of 7600, compatible with the presence of 66 amino acid residues per molecule. The N-terminal amino acid sequence was determined (up to residue 48) and showed approximately 95% similarity with toxins from other Mexican scorpions of the genus *Centruroides*. Experiments conducted with chick dorsal root ganglion cells showed that toxin I is a Na^+ channel effector, causing a decrease in the peak Na^+ permeability, similar to decreases observed for typical β-scorpion toxins.

INTRODUCTION

SCORPIONISM is an important health problem in the city of Leon, state of Guanajuato (Mexico). The only dangerous scorpion reported in this area is *Centruroides infamatus infamatus* (Cii) from the Buthidae family. It is a slender yellow scorpion: 6–8 cm in length. It cohabits with humans, causing a high incidence of scorpion stings, as previously reported (DEHESA-DÁVILA, 1989; DEHESA-DÁVILA and POSSANI, 1994), and to the best of our knowledge only an abstract (DEHESA-DÁVILA and POSSANI, 1990) is available in the literature, concerning the biochemistry of its venom. During the years 1990–1993 there were 35,777 cases of scorpion stings registered (personal communication, Dr CHAVEZ HARO). Horse antiserum (Alacramyn™†) has successfully been applied to 18,212

* Author to whom correspondence should be addressed.
† Alacramyn is a trade mark from Laboratorios Biocion, Calz. Tlalpan 4687, México D.F. 14050, México.

people, reducing the mortality rate to zero in the Mexican Red Cross Hospital of Leon.

In this communication we report the isolation and the N-terminal amino acid sequence of a toxin from this scorpion species (Cii), and its effect on Na^+ channels of chick dorsal root ganglion (DRG) cells.

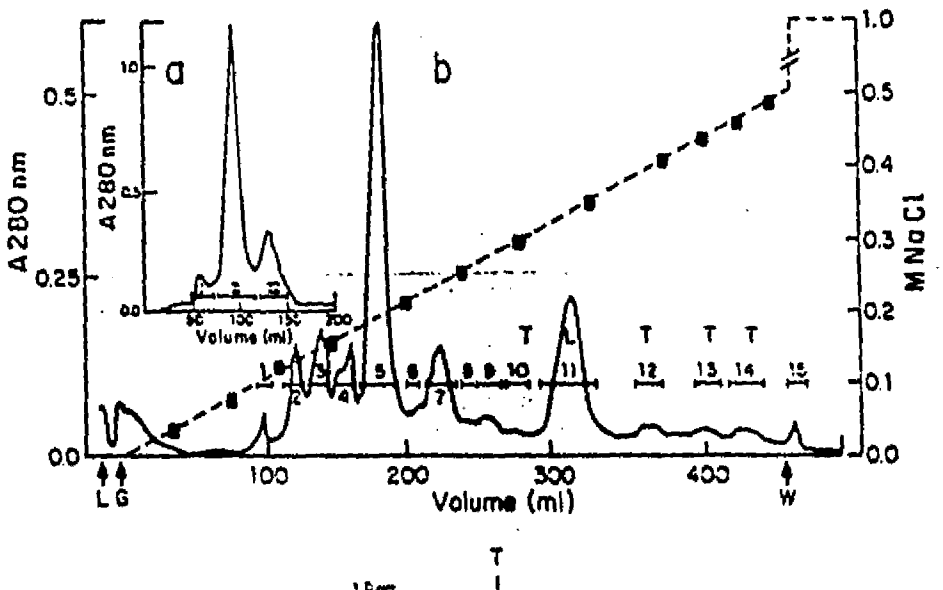

FIG. 1. CHROMATOGRAPHIC SEPARATION OF Cii VENOM.

(a) Cii soluble venom (21.8 mg by absorbance at 280 nm) in 2 ml solution was applied to a Sephadex G-50-medium column (200 × 0.9 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7. The flow rate was 25 ml/hr and fractions of 2.5 ml were collected and pooled according to their absorbance at 280 nm, as indicated by the horizontal bars. Only fraction II was toxic to mammals. T, Toxic. Columns were run at room temperature (22° C). (b) Fraction II from independent applications on the Sephadex G-50 (37.5 mg total) was applied to a CMC-32 (0.9 cm × 30 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 30 ml/hr. A linear salt gradient in 500 ml total volume, from 0 M to 0.5 M NaCl (250 ml each) was used to elute the column. L, Loading the sample; G, starting point of the gradient; W, washing with 1 M NaCl. (c) Fraction II-11 (8.6 mg) was applied to the same column as in (b) equilibrated and eluted in 50 mM K^+ phosphate buffer, pH 6.0, using a linear gradient from 0 to 0.4 M NaCl in the same buffer. (d) HPLC purification of fraction II-11.1 in a C18 reverse phase column (Vydac, Hysperia, CA, U.S.A.), using a Beckman 421A chromatographic system with a linear gradient from 0 to 60% of acetonitrile, in the presence of 0.1% trifluoroacetic acid

MATERIALS AND METHODS

The scorpions were collected by us in the North of the city of Leon, Guanajuato. The venom was obtained by electrical stimulation of anaesthetized animals as previously described (DENT *et al.*, 1980). Water-solubilized venom was centrifuged at 16,000 × *g* for 15 min in a Sorvall SS-34 rotor. The supernatant was freeze-dried and stored at −20°C until used.

The mouse lethality of various protein fractions was observed after i.p. injection of different amounts of protein (usually from 10 to 50 μg) in 0.2–0.4 ml saline or buffer solutions, into adult 20–25 g mice (strain CD1). Lethality test were conducted with a minimum of animals according to protocols approved by the Animal Care Committee of our Institute. To define the toxicity of various chromatographic components, three designations were used (POSSANI *et al.*, 1977). 'Lethal' means that the component at the dose injected was enough to kill the tested mouse within 4 hr of injection. 'Toxic' means that the mouse shows any of the following symptoms: excitability, salivation, temporary paralysis of rear limbs, dyspnea, but recovered within 20 hr after injection. 'Non-toxic' means normal behaviour similar to that after the injection of 0.9% NaCl or buffer solutions. The LD_{50} value of the toxic fraction II, from Sephadex G-50, was determined graphically (POSSANI *et al.*, 1977) using five different protein concentrations of this fraction. The LD_{50} was estimated to be 1.27 mg/kg for CD1 strain of albino mice. The protein contents of venom and chromatographic subfractions of venom were calculated based on absorbance at 280 nm, assuming 1 unit of absorbance, in a 1 cm pathway cuvette, equal to 1 mg/ml protein.

RESULTS

The soluble venom (21.8 mg) was applied to a Sephadex G-50-medium column (200 cm × 0.9 cm). The toxin-containing tubes were pooled (fraction number II) and further separated on a carboxy-methyl-cellulose resin (CMC-32, from Whatman, Springfield, U.K.), equilibrated and run in the presence of 20 mM ammonium acetate buffer, pH 4.7. The major toxin fraction obtained (II.11) was subsequently purified by two additional steps: first on CMC-32 column equilibrated with 50 mM K^+-phosphate buffer, pH 6.0; and second, with high-performance liquid chromatography (HPLC) through a C18 reverse phase column. Additional details on the purification procedures are indicated in the legend of Fig. 1. When necessary the fractions were dialysed against the appropriate buffers, using a Spectrapor 3 M dialysis membrane (Spectrum Medical Industries, LA, CA, U.S.A.). The purity of the fractions was assessed by polyacrylamide gel electrophoresis (PAGE) using the system described by REISFELD *et al.* (1962).

Figure 1(a) shows a chromatographic profile of the venom separation through Sephadex G-50-medium chromatography. This gel filtration provided three fractions in a very reproducible manner (this step was repeated at least five times). Fraction II corresponds to 73% of the soluble venom, and was lethal to mice. Fractions I and III were not toxic at the dose assayed (50 μg/mouse). Overall recovery from Fig. 1(a) was 98% (Table 1). Fraction II from Sephadex G-50 applied to a CMC-32 column separated 15 subfractions [Fig. 1(b)] and the final recovery was 99%. Component II.15 was obtained by washing the column with 1 M NaCl and was shown to be toxic only to crustaceans. Fraction II.11 represents 16% of the material recovered, and was re-applied to a CMC-32 column, as indicated in Fig. 1(c). The principal toxic component (II-11.1) was subsequently purified by HPLC, as shown in Fig. 1(d). Component number 1 (label T) was lethal to mice and run as a single band (data not shown) on PAGE (REISFELD *et al.*, 1962). The apparent mol. wt estimated from sodium dodecyl sulfate-PAGE (LAEMMLI, 1970) was in the range of 7600; hence, compatible with the presence of approximately 66 amino acid residues per molecule of peptide. A sample of this toxin (150 μg) was reduced and carboxymethylated (RC-toxin), as previously described (MARTIN *et al.*, 1988). RC-toxin (1.0 nmol) was attached to a Sequelon-AA membrane and sequenced using a MilliGen-Biosearch Prosequencer.

TABLE 1. RECOVERY AND LETHALITY OF CHROMATOGRAPHIC FRACTIONS

Column	Fraction	Protein content (mg)*	Recovery (%)	Lethality (LD_{50})
Sephadex G-50	Soluble venom	21.8	100	Lethal
(Fig. 1a)	Fraction I	1.1	5	Non-toxic
	Fraction II	16.0	73	(1.27 mg/kg)
	Fraction III	4.3	20	Non-toxic
	Total	21.4	98	
CMC-32	Fraction II	37.5	100	Lethal
(Fig. 1b)	Fraction II.1	0.6	5	Not tested
	Fraction II.2	1.9	5	Not tested
	Fraction II.3	2.8	7	Not tested
	Fraction II.4	3.1	8	Not tested
	Fraction II.5	10.6	28	Non-toxic
	Fraction II.6	1.7	5	Non-toxic
	Fraction II.7	3.1	8	Non-toxic
	Fraction II.8	0.6	2	Non-toxic
	Fraction II.9	1.0	3	Toxic
	Fraction II.10	0.6	2	Toxic
	Fraction II.11	6.0	16	Lethal
	Fraction II.12	1.3	3	Toxic
	Fraction II.13	1.1	3	Toxic
	Fraction II.14	0.4	1	Toxic
	Fraction II.15	0.4	1	Toxic
	Side tubes	1.8	5	Not tested
	Total	37	99	

*The values reported are percentages, based on absorbance units at 280 nm. Lethality tested were performed in CD1 albino mice, with a dose of about 50 μg protein per mouse.

The amino acid sequence found was: Lys-Glu-Gly-Tyr-Leu-Val-Asn-His-Ser-Thr-Gly-Cys-Lys-Tyr-Glu-Cys-Tyr-Lys-Leu-Gly-Asp-Asn-Asp-Tyr-Cys-Leu-Arg-Glu-Cys-Lys-Gln-Gln-Tyr-Gly-Lys-Gly-Ala-Lys-Gly-Tyr-Lys-Tyr-Ala-Phe-Gly-Cys-Trp-Cys . . .

We propose calling this peptide: toxin 1 from *Centruroides infamatus infamatus* (abbreviated Cii-1). The N-terminal amino acid sequence obtained (through residue 48) was compared with known toxins from scorpions of the genus *Centruroides*, showing a striking degree of similarities. Comparing the sequenced portion of Cii-1 (about two-thirds of the full length peptide) with that of *C. limpidus tecomanus* toxin 1 (MARTIN *et al.*, 1988) showed 96% identity, *C. l. limpidus* (RAMIREZ *et al.*, 1994) toxin 1 had 94% identity, while *C. noxius* toxin 4 (VÁZQUEZ *et al.*, 1993) and *C. suffusus suffusus* toxin II (ROCHAT *et al.*, 1979) had 94% and 87% identity, respectively. The least similar was variant 2, from *C. sculpturatus* (BABIN *et al.*, 1975), which showed only 67% identity with Cii-1. Thus, all these peptides from the genus *Centruroides* are definitively related.

In order to verify the function of Cii-1 we have assayed the pure toxin on chick dorsal root ganglion cells. The effect of toxin Cii-1 (at 0.5 μM concentration) on these cells demonstrates that it affects Na^+ permeability [Fig. 2(b)]. The experiments were performed with primary cultured neurons from dorsal root ganglion of 10-day-old chick embryo. Sensory neurons were grown as described (BARDE *et al.*, 1980) and current recordings (HAMILL *et al.*, 1981) were obtained in the whole cell configuration system, at room temperature (20–22°C). The experimental set-up previously described by us (NOBILE *et al.*, 1990) was used. Membrane currents were evoked by voltage steps of 10 mV, 40 msec long, from −40 to 50 mV, and the holding potential (VH) was kept at −60 mV. Data acquisition and voltage pulses generation were performed on line with a PDP 11-23 minicomputer. Membrane currents were filtered at 3 kHz, digitized with a sampling

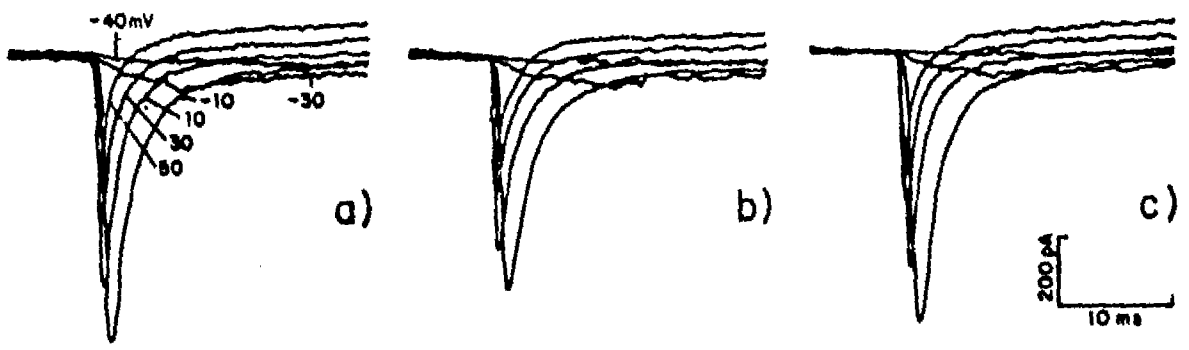

FIG. 2. ELECTROPHYSIOLOGICAL RECORDINGS ON DRG CELLS.
(a) A family of currents, due to step depolarization from −60 mV to +50 mV, evoked in a voltage-clamped cell. External and internal solutions were chosen in order to eliminate K^+ and Ca^+ currents. The composition of the external solution was (meq): 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 20 glucose, 10 HEPES, 1 CdCl. The pipette filling solution contained (meq): 120 CsCl, 20 tetra-ethyl-ammonium choride, 10 EGTA, 2 $MgCl_2$, 10 HEPES. Solutions were adjusted to pH 7.3, with NaOH (external solution) or CsOH (internal solution) and adjusted also to 300 mosm/kg by adding mannitol. (b) Action of 0.5 μM toxin Cii-1 on Na^+ currents at the membrane potential indicated in (a). The peak Na^+ current decreased by about 25%. (c) Na^+ currents after washing the cell with toxin-free solution. Recovery was almost completed after 2 min washing.

frequency of 10 kHz and stored for further analysis. In all experiments series resistance compensation for the pipette resistance was used to obtain maximal response time constants. Leakage and capacitive current components were reduced by appropriately subtracting scaled currents. Figure 2(a) shows a family of currents, due to step depolarization from −40 mV to +50 mV, evoked in a voltage clamped cell. A fast transient inward current was present at all potentials; however, an opposite residual outward current appeared occasionally at membrane potentials more positive than 0 mV, very likely due to Cs^+ ions passing through K^+ channels (ionic conditions in the legend for Fig. 2). The fast current disappeared following the addition of 3 μM tetrodotoxin into the bath or by replacing the external Na^+ with choline (data not shown). This indicates that inward currents were due to Na^+ ions. Figure 2(b) shows the effect of Cii-1 externally applied to the same cell. A decrease in the Na^+ current of approximately 25% was observed. Qualitatively similar results were recorded in about 15 more neurons assayed. Figure 2(c) shows the results of washing the plate with buffer without toxin. Almost

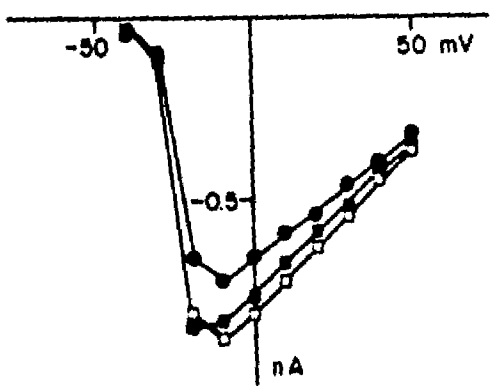

FIG. 3. CURRENT-VOLTAGE RELATIONSHIP SHOWING THE REVERSIBLE EFFECT OF Cii-1.
Empty square represent control conditions, full circles indicate the action of toxin Cii-1, and full squares, the recovery to the initial conditions. This graphic was obtained from the family currents described in Fig. 2.

complete recovery was obtained after 2 min. A summarized current–voltage curve of this experiment is shown in Fig. 3.

DISCUSSION

We report the purification of a new toxin (Cii-1) from the venom of the scorpion *Centruroides infamatus infamatus*, which is the first to be described from this venom. The N-terminal amino acid sequence is highly similar to toxins extracted from other Mexican scorpions, as expected. This toxin is a Na^+ channel effector, causing a decrease in the peak Na^+ permeability, similar to that observed for typical β-scorpion toxins from the New World (YATANI *et al.*, 1988). This effect is reversible, under washing (Fig. 2c). Thus, toxin Cii-1 is another β-scorpion toxin that increases the vast repertoire of known peptides evolved by scorpions, and should serve as another variant for structure–function relationship studies of toxin–channel interactions.

One of the most interesting questions arose when we compared the effect of Cii-1 with that of *C. l. tecomanus* toxin 1 in the same DRG cells (MARTIN *et al.*, 1988). Applied to DRG cells, at the same concentration, both toxins have a relatively small effect (not higher than 25% change), but the mechanism of action appears to be different. While *C. l. tecomanus* toxin 1 seems to slow inactivation of Na^+ channels in DRG cells, similar to the Old World scorpion toxins (see MARTIN *et al.*, 1988), *C. l. infamatus* toxin 1 (this communication) seems to decrease the peak permeability of Na^+ currents. Thus, these two toxins might be important tools for studying Na^+ channel function versus toxin structure, especially when the full amino acid sequence of Cii-1 becomes available.

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Anexo 1

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ANEXO 2

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SCORPIONISM AND SEROTHERAPY IN MEXICO

MANUEL DEHESA-DÁVILA and LOURIVAL D. POSSANI*
Instituto de Biotecnologia, Universidad Nacional Autónoma de México, Av. Universidad 2001, Apartado Postal 510-3, Cuernavaca, Morelos 62271, Mexico

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M. DEHESA-DÁVILA and L. D. POSSANI. Scorpionism and serotherapy in Mexico. *Toxicon* **32**, 1015–1018, 1994.—In Mexico, scorpionism is an endemic public health problem. The exact number of human accidents is unknown, but partial statistics suggests numbers close to 200,000 per year. The documented number of fatality cases is in the order of 310 people per year. We currently use horse antiserum in patients who show a clear picture of intoxication. Our personal experience in treating 38,068 people, from which over 20,000 received serotherapy, shows that the antiserum is very effective, in that none of the patients died.

IN MEXICO, about 134 different scorpion species (including subspecies) have been found, but only eight of them are dangerous to humans, and are responsible for the majority of stings. They belong to the genus *Centruroides*, family Buthidae. Hoffmann described at least 30 different species and subspecies in this genus (HOFFMANN, 1936; HOFFMANN and NIETO, 1939). The dangerous species are: *Centruroides elegans*, *C. infamatus infamatus*, *C. limpidus limpidus*, *C. l. tecomanus*, *C. noxius*, *C. pallidiceps*, *C. sculpturatus* and *C. suffusus suffusus*. These arachnids are found on the Pacific Coast, where 11 Mexican states were shown to be endemic places for scorpions, and consequently responsible for a hazardous public health problem.

Unfortunately, the epidemiological data are incomplete and the exact number of accidents is unknown. Original estimates of the number of cases were published by MAZZOTI and BRAVO-BECHERELLE (1963) and reported by MONROY-VELAZCO (1961). Numbers as high as 100,000 cases of accidents per year were reported during the 1960s and 1970s. LOPEZ-ACUÑA and ALAGON have compiled data (presented in the technical session of the Sociedad Mexicana de Salud Publica) for 1976 suggesting numbers close to 200,000 per year. In 1989, DEHESA-DÁVILA reported the incidence of 38,068 cases of people stung in Leon, registered only at the Red Cross Hospital, during the period 1981–1986.

In this discussion (see Table 1) we include unpublished data from statistics collected by us in the city of Leon, Guanajuato, where one of us (MDD) worked, giving clinical attention to patients during the period of 1984 to 1986. Also included are partial data from two other cities: Cuernavaca in Morelos State, and Tepic in the State of Nayarit, compiled

*Author to whom correspondence should be addressed.

TABLE 1. PARTIAL STATISTICAL DATA OF SCORPION STINGS IN MEXICO

Year	Leon*	Cuernavaca	Tepic	Total national mortality†
1981	6649	n.c.‡	n.c.	390
1982	8044	n.c.	n.c.	401
1983	7342	1759	1759	345
1984	7928	1564	1068	312
1985	7289	2641	1063	291
1986	7989	3042	1206	278
1987	7155	3775	1510	272
1988	7130	3661	1109	290
1989	9279	n.c.	1814	233
1990	9373	n.c.	n.c.	285
1991	9587	n.c.	n.c.	n.c.
1992	8671	n.c.	n.c.	n.c.
Total	96,436	16,442	9529	3097
Global	122,407			

* Only Red Cross Hospital and one clinic of IMSS (Mexican Institute of Social Security) in Leon, Guanajuato.

† Mortality officially registered by the Ministry of Health for the entire country.

‡ n.c., Not compiled.

by us for comparative purposes. We have documented 122,407 accidents during 12 years of observation in Leon, 6 in Cuernavaca and 7 in Tepic. The city of Leon alone had approximately 8000 stings per year during this time. Yet these data were recorded only from the Red Cross Hospital and one of the Social Security Hospitals. Also, the data collected in Cuernavaca and Tepic refer only to the Hospitals of the Health Ministry (Secretaria de Salud). These cities have other hospitals. The actual documented cases in Table 1 indicate a mean value of about 11,500 accidents per year, in those three cities alone. There are other factors to be considered, such as cases where people (healthy adults) do not always go to the hospital when stung by scorpions. These data taken together indicate that the number of accidents by scorpions in Mexico is high. The number of fatal accidents in the entire country is also considerably high, about 310 people per year (Table 1). However, the official statistics are surely underestimated by a factor of 2 or 3. In many cases, the certificate of death (*causa mortis*) states that the patient died because of a cardiocirculatory shock, pulmonary oedema or cardiac arrest, but does not associate the scorpion sting to these events.

The envenomation and prognosis will depend on a number of factors, some of them attributable to the scorpion and some to the victim. For the scorpion these are: (1) species, (2) condition of the telson (stinger) at the moment of the accident, and (3) the number of stings and quantity of venom injected. For the victim: (1) age, weight and health of the victim, (2) concomitant diseases (diabetes, hypertension, heart disease, etc.), and (3) effectiveness of the treatment. From the onset of the first signs and symptoms of envenomation to the development of a severe picture might take a very short time; in most cases this progression can take only 5 to 30 min. Serious systemic complications such as heart failure, pulmonary oedema, circulatory shock, convulsions and coma worsen the prognosis. Respiratory failure is usually the cause of death, but other severe complications like cardiocirculatory shock may lead to death (DEHESA-DÁVILA, 1989; DEL POZO and GONZALEZ, 1945).

The most important points, for the purpose of this discussion, however, are the clinical events and the serotherapeutic treatment applied to the patients. The physicians (or trained

paramedicals) can easily distinguish two distinct symptoms (local and general) following a scorpion sting.

Local symptoms

Immediately after the sting there is burning pain, which is followed by pruritus and hyperaesthesia. Mild inflammation sometimes occurs.

General symptoms

The following list of symptoms is not always observed and they do not have an apparent sequence: hyperexcitability, restlessness, hyperthermia, tachypnoea, dyspnoea, tachycardia or bradycardia, profuse sweating, nausea, vomiting, gastric hyperdistention, diarrhoea, lachrymation, nystagmus, mydriasis, photophobia, excessive salivation, nasal secretion, dysphagia with a sensation of hair in the throat, dysphonia, cough, bronchorrhoea, pulmonary oedema, arterial hypertension or hypotension, heart failure, circulatory shock, convulsions, ataxia, fasciculations and coma.

Most of this symptomatology is due to the stimulation of the autonomic nervous system which includes parasympathetic (cholinergic) and sympathetic (adrenergic) pathways. Both are stimulated by scorpion venom. In the course of the envenomation one effect can be predominant, but mixed effects are also observed. This is the reason for finding variable symptomatology as discussed by Freire Maia *et al.* (1976), and Freire Maia and Campos (1989). Another factor to be taken into consideration, as demonstrated by Ismail (1988), is the rapid distribution of the venom and the time for its elimination from the body. The intravenous route dissemination of the venom component ranges from 4 to 7 min and the overall elimination half-lives are from 4.2 to 13.4 hr.

In our opinion, the key objective of the physician at this stage is to be able to neutralize the circulating venom with antiserum, as soon as possible, when the patient needs this treatment. However, this is not a trivial decision. Usually, in the cases treated by us, when the patient manifests only the local symptoms *we do not* apply the serum. If the patient presents two or more of the general symptoms described above *we do* apply the serum, immediately. In several years of clinical practice in Leon (Dehesa-Dávila, 1989), by following this procedure and using the sera mentioned below (Alacramyn®), none of our patients died. From 38,068 patients that came to the Red Cross Hospital, antiserum was administered to 20,293. Skin tests, or other procedures, were not performed to determine possible hypersensitivities. None of the patients developed immediate allergic reaction. Cases of late development of serum sickness, although possible, were not documented, mainly because the patients do not return to the hospital. Patients arriving late at the hospital with complicated symptoms are also treated immediately with serum, and then placed into special care units for adequate monitoring and other drug-therapy treatments, according to the clinical symptomatology manifested.

In Mexico, *Centruroides* polyvalent antivenom is produced in horses by injecting with a mixture of macerates of venomous gland from the most important species (*C. noxius*, *C. l. limpidus*, *C. l. tecomanus*, and *C. suffusus suffusus*). It is enzymatically digested and lyophilized. The antivenom cross-reacts with other *Centruroides*, and protects against all venomous species of the country. There are two producers of antiserum against *Centruroides* species:

(1) Gerencia General de Biologicos y Reactivos, Health Ministry, M. Escobedo 20, C.P. 11400 Mexico D.F., Mexico. Suero Antialacran—ampoule with lyophilized immunoglobulins. One ampoule neutralizes 150 LD_{50} in mice tested intraperitoneally.

Anexo 2

(2) Laboratorios BIOCLON S.A. (before MYN, Zapata, and Grupo Pharma). Calzada de Tlalpan 4687, C.P. 14050 Mexico D.F., Mexico. Suero Antialacran (Alacramyn)*—ampoule with lyophilized immunoglobulins. One ampoule neutralizes 150 LD_{50} in mice tested intraperitoneally.

The quantity of antivenom to be used is determined by the clinical symptoms and their evolution in time. The lyophilized immunoglobulins contained in the ampoule are dissolved in 5 ml of water for injection. In earlier stages of envenomation, one or two vials are enough to neutralize the circulating venom, hence controlling the clinical symptoms. However, when the patient comes to the hospital in later stages of envenomation it might be necessary to use up to four vials of serum. This is valid for both children and adults. It is always advisable to administer an antihistamine (i.e. chlorpheniramine) together with antiserum. Faster neutralizing effects are obtained if the intravenous route of injection is used. Intramuscular injection of the antiserum is also effective but its absorption is slower. The use of the antiserum is not recommended in the absence of systemic symptoms of envenomation.

The conclusion of this work is that there is no doubt that the application of immune serum is the most important therapeutic measure to neutralize the circulating venom. We are not neglecting the appropriate treatment of the clinical symptoms with other available drugs and supportive therapies, when required. Another important fact is the use of potent (high-titre) and polyvalent antisera for serotherapy. The latter factor might explain some disappointing results obtained by other people in other parts of the world, where these factors were not taken into consideration, or even worse, where a serum with these characteristics is unfortunately not available (GUERON *et al.*, 1993; ISMAIL, 1993).

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ANEXO 3

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Mr. Lourival D. Possani, Ph.D.
Instituto de Biotecnologia
Dept. Biochemistry
Universidad Nacional Autonoma
de Mexico
Apdo. 510-3, Morelos 62271

Cuernavaca / Mexico

August 16, 1994

Dear Mr. Possani

I wirte to you on behalf of Dr. Jürg Meier to inform you that the manuscript will be sent to the publishers by the end of September. Please find enclosed the copy for Manuel Dehesa as requested.

Yours sincerely

A. Stöcklin, secretary to Dr. Meier

CLINICAL TOXICOLOGY OF ANIMAL VENOMS AND POISONS
A Newsletter to all Authors

April 10, 1995

Dear Colleagues,

there we are finally ! The book to which you contributed considerably with your excellent chapter(s) on particular aspects of clinical toxicology of animal venoms and poisons is now in its final stage of preparation. The index is presently under preparation and should be finished within ten days time.

You should receive the „final" draft copies, as soon as we receive them from CRC Press (the manuscripts have been provided in „camera ready format") within the next 4 to 6 weeks. We are convinced that the book will be on the market probably early fall 1995.

We are wondering whether CRC Press is able to keep its promise to publish the book for less than US$ 100.00 ! This would really make the book a „best seller" in Toxinology.

On behalf of Dr. Julian White and myself, I wish to thank you at this stage for your very valuable contribution. At the same time, let us apologize for our long silence. However, the editorial work was also for us some sort of a „late night" and „weekend" event besides our regular work.

With all best wishes,

Yours sincerely

Dr. Julian White,
Adelaide
Australia

Dr. Jürg Meier
Basel
Switzerland

Chapter 18

CLINICAL TOXICOLOGY OF SCORPION STINGS

Manuel Dehesa-Davila, Alejandro C. Alagon and Lourival D. Possani

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IX. References.

I. INTRODUCTION

This chapter encompasses the clinical toxicology of scorpion stings, using as a model the scorpions from the genus *Centruroides* of Mexico. Statistical estimates indicate that in Mexico about a quarter of a million sting accidents occur in humans every year. Also, due to the diversity of climates, about 136 different species and sub-species of scorpions are endemic in Mexico.[1] Fortunately only eight species and sub-species are clinically important for man.[1,2]

Scorpions are arachnids well distributed geographically, in tropical and sub-tropical areas of the world, and possess a venom apparatus composed of a pair of glands and a sting. The species important for human health contain in their venoms small molecular weight peptides capable of causing impairment of cell function by interfering with ion channel permeability of the membranes of excitable cells.

This chapter we will present and discuss some of the general aspects of the venom, symptoms and signs of envenomation, prophylaxis and clinical management, based on the author's own experience; especially in the treatment of persons stung by scorpions. More than ten thousand cases were treated by our group in different clinical facilities. Some references are also included concerning scorpions in other parts of the world.

II. EPIDEMIOLOGY OF SCORPION STINGS IN MEXICO

A. THE SCORPIONS: DISTRIBUTION AND CHARACTERISTICS:

Worldwide, there are more than 1,000 scorpion species distributed in six families: Buthidae, Scorpionidae, Diplocentridae, Chactidae, Vejovidae and Bothriuridae.[3] Although all scorpions are venomous, less than 50 species (belonging to the Buthidae family), can be considered dangerous to humans.[3]

In Mexico, the only species of scorpions of medical importance are from the genus *Centruroides*, which belongs to the Buthidae family. There are at least 30 different species and subspecies from this genera described by Hoffmann.[1,2]

The scorpions are arthropods that belong to the class Arachnida and to the order Scorpionida. They have two main body divisions: the prosoma or cephalothorax and the opisthosoma or abdomen. The abdomen is further subdivided into an anterior mesosoma (preabdomen) and a posterior metasoma (postabdomen or "tail"). Both of these regions are segmented. The tail-like metasoma terminates in a bulbous segment called the telson. This structure possesses a sharp, curved stinger or aculeus. A scorpion is equipped with a pair of pedipalps that look like claws; the small chelicerae between the bases of the pedipalps; four pairs of legs, all on the prosoma; and a pair of pectines, which are ventrally located, have comb-like structure and are sensorial organs.[3] For additional information on the morphology of scorpion, please refer to Chapter __ of this series.

Centruroides scorpions belong to a New World genus with its center of distribution in Mexico (Fig.1). These scorpions are also found in the southern states of the United States, Central America, the West Indies and in several countries of South America. Specifically, the species of medical importance occur in Western New Mexico, Arizona, areas along the basin of the Colorado river in California, and in Mexico, in the Pacific States from the North of Sonora down to the West-side of Oaxaca and in some regions of some Central States such as Durango, Aguascalientes, Zacatecas, Guanajuato and Morelos.[4] In Fig.2 illustrates representatives species of Mexican scorpions.

Scientific name: *Centruroides*
Species (States in Mexico, or U.S.A.)

Centruroides bertholdi (Jalisco)
•*Centruroides elegans* (Nayarit, Jalisco, Michoacán, Colima, Guerrero)
Centruroides exilicauda (Sonora, Baja California Norte, California in USA)
Centruroides flavopictus (Veracruz)
Centruroides fulvipes (Guerrero)
•*Centruroides infamatus infamatus* (Zacatecas, Aguascalientes, Guanajuato, Michoacan)
•*Centruroides limpidus limpidus* (Colima, Guerrero, Morelos)
•*Centruroides limpidus tecomanus* (Colima)
Centruroides margaritatus (Oaxaca)
Centruroides nigrescens (Oaxaca)
Centruroides nigrovariatus (Oaxaca)
•*Centruroides noxius* (Nayarit)
•*Centruroides pallidiceps* (Sonora, Sinaloa, Arizona in USA)
•*Centruroides sculpturatus* (Sonora; Arizona and New Mexico in USA)
•*Centruroides suffusus suffusus* (Durango)

The most dangerous species are highlighted with an asterisk (•). See also the map of geographical distribution in Fig.1. In Mexico the dangerous species are distributed along the Pacific coast, comprising 16 States of the country, while in the U.S.A the dangerous species occur mainly in Arizona and New Mexico. The morphological characteristics of *Centuroides* sp., including the most danderous species, are listed in Chapter 17.

B. SCORPION STINGS IN MEXICO

Scorpionism is a public health problem in many states of Mexico. Unfortunately, the epidemiological data are incomplete and the exact number of accidents is unknown. Original estimates on the number of cases were published by Mazzotti and Bravo-Becherelle[6] and reported by Monroy-Velazco.[7] Numbers as high as 100,000 cases of accidents per year were reported for the years 60's and 70's. Lopez-Acuña & Alagon have compiled data (presented in the technical session of the Sociedad Mexicana de Salud Publica) in 1976 suggesting numbers close to 200,000 per year, with 700 to 800 fatalities. Dehesa-Davila[8] and Possani et al.[9] confirm estimates higher than 250,000 stings per year in Mexico. Table 1 summarises unpublished data from statistics collected by the authors in the city of Leon, Guanajuato. Also included are partial data from the states of Nayarit and Morelos. There have been about 69,000 accidents in 9 years of observation. The city of Leon alone had approximately 7,540 stings per year (data recorded only from the Red Cross Hospital and one of the Social Security Clinics of the city), during this period. Yet, the city of Leon is not the most important in Mexico in terms of scorpionism.[8] The mortality rate is 0.01%.

III. VENOM COMPOSITION AND PHARMACOLOGY

A. STRUCTURE AND MODE OF ACTION OF SCORPION TOXINS

In humans and experimental animals toxic polypeptides of scorpion venom are responsible for the symptoms of intoxication. There are two groups of similar peptides in the venoms of *Centruroides*: one is composed of long-chain peptides that affect sodium channels, and the second consists of short-chain peptides which block potassium channels of excitable membranes (mainly nervous and muscular tissues). The binding of these toxins to the cation channels (Na^+ and K^+) causes most of the toxicological symptoms of the venom. The binding is reversible, but different toxins have different affinities.[10-15] Recently, another class of peptides was described in the venom of the scorpion *Buthotus hottentota*, which affects the Ryanodine-sensitive Ca^{2+}-channel[16], and similar peptides were found in the venom of the genus *Hadrurus* from Mexico.[17] The literature of the last five years reflects the importance of the structure and function relationship of toxins from scorpions collected in many different parts of the world.[18-23] Fig. 3 is an example of the primary structure of toxins isolated from scorpions of the genus *Centruroides*. In this figure both classes of scorpion toxins are represented: long-chain (Na^+-channel blockers) and short-chain (K^+-channel blockers).

B. HUMAN DATA

It is only possible to obtain a rough estimate concerning the amounts of venom that result in envenomation in humans. This approximation is based on the amount of venom extracted by electrical stimulation from the telson of anaesthetized scorpions. The average amount of venom that can be obtained from a single animal varies from 100 µg in small species (i.e. *Centruroides noxius*) to 600 µg in the large species (i.e. *Centruroides elegans*). These experimental values set the range of variation on the amount of venom toxic to humans. It is unlikely that a scorpion in a normal situation will inject more venom than is extracted under extreme stress (electrical stimulation). Scorpions usually inject venom subcutaneously, from where it is distributed all over the body by the circulatory system.
The human lethal dose is probably less than 100 to 600 µg of venom. This is the maximum amount of venom that a single scorpion has in the glands (telson) at any one time.
Ismail[24] demonstrated that the venom is distributed very rapidly into the tissues by the intravenous route ranging between 4 - 7 minutes and overall elimination half-lives of 4.2 to 13.4 hr.

C. ANIMAL DATA

LD_{50} values determined in experimental animals vary according to the species and strain of animal used. For example the LD_{50} estimated for *Centruroides limpidus limpidus* venom in seven different strains of mice averaged (intraperitoneal injection) at 2 mg/kg mouse with the extremes of variation being 0.61 mg/kg for the SSA strain and 3.31 mg/kg for the BALB/k strain.[25] The LD_{50} of *Centruroides infamatus infamatus* venom injected intraperitoneally into mice was 1.27 mg/kg, but was only 0.166 mg/kg in Wistar strain rats using the same route. *Centruroides noxius* seems to be the most toxic venom, with an LD_{50} of 0.26 mg/Kg mouse intraperitoneally.[26] *Centruroides limpidus tecomanus* has an LD50 of 0.65 mg/kg, while that of *Centruroides sculpturatus* is 1.12 mg/kg in mice injected intraperitoneally.[27]

1. IN VITRO DATA

Most of the data on the mechanism of action of the toxins from the scorpion venoms were obtained by *in vitro* experiments using excitable tissues such as squid axons, several types of heart cells, dorsal root ganglion cells, synaptosomes from brain tissues, and others. Whole-cell clamp and patch-clamp techniques, or single channels incorporated into artificial bilayer membranes were used to identify the channels affected by toxins purified from the venom of *Centruroides* scorpions.[12,28-31]

2. ELIMINATION BY ROUTE OF EXPOSURE

Elimination of venom occurs mainly through urinary excretion, although bile secretion may play an important role.[32] People stung by *Centruroides* scorpions may have clinical symptoms for as long as one week, especially near or at the site of the sting (unpublished observations).

IV. SYMPTOMS AND SIGNS

A. GENERAL COMMENTS

Centruroides scorpions have a tendency to hide above the ground, therefore they are frequently found under loose bark on trees, crevices in dead trees, logs and walls or at the base in dry leaves of palms and corn plants. They may also thrive in lumber piles, bricks, stones and other debris or in soil cracks. Poorly built houses offer scorpions many niches to live. Indeed, they are often associated with human habitations because they enter homes and hide during daylight hours in places offering darkness and close contact (shoes, folded blankets, heaped clothing, hanging pictures, etc). Scorpions are almost exclusively nocturnal. *Centruroides* scorpions are not aggressive; accidents occur when they are inadvertently touched in their hiding places or when they are wandering in search of food. In Mexico, there is an increase in the morbidity and mortality rate that in some places coincides with the rainy season and in others with the beginning of spring. The most dangerous *Centruroides* species dislike damp places preferring dry indoor locations.[33]

All species belonging to the *Centruroides* genus or other genera, dangerous or harmless are called "scorpions" in English speaking countries, and "escorpiones" or "alacranes" in Spanish speaking countries.

RISK FACTORS

Envenomation and prognosis will depend on a number of factors, some of them attributable to the arthropod and some to the victim. For the arthropod these are: (i) species (ii) condition of the telson at the moment of the sting (iii) number of stings and quantity of venom injected. For the victim: (i) age, weight and health of the victim (ii) concomitant disease (i.e. diabetes, hypertension, heart disease, etc.) and (iii) effectiveness of the treatment. From the onset of the first signs and symptoms of envenomation to the development of severe envenoming may take a very short time; in most cases this progression can take only 5 to 30 minutes. Prognosis: serious systemic complications such as heart failure, pulmonary oedema, convulsions and coma worsen the prognosis. Respiratory failure is usually the cause of death, but other severe complications, like cardiocirculatory shock may lead to death.[8,34]

B. ONSET, EVOLUTION AND CLINICAL PICTURE

Local evidence of the sting in the skin of the victim is often minimal or absent. Patients report intense pain or a burning sensation with intense pruritus and hyperesthesia. Some

redness and inflammation with local oedema can be observed at the sting site. The pain occurs instantaneously after the sting and can last for several days; paresthesia can be present for several weeks and is usually the last symptom to disappear.

a) Local symptoms and signs.
Immediately after the sting there is burning pain, which is followed by pruritus and hyperaesthesia. Mild inflammation sometimes occurs.

b) General symptoms and signs.
General symptoms are not always observed and do not have an apparent sequence. They include some or all of the following: hyperexcitability, restlessness, hyperthermia, tachypnoea, dyspnoea, tachycardia or bradycardia, profuse sweating, nausea, vomiting, gastric hyperdistention, diarrhoea, lachrymation, nystagmus, mydriasis, photophobia, excessive salivation, nasal secretion, dysphagia with a sensation of hair in the throat, dysphonia, cough, bronchorrhea, pulmonary oedema, arterial hypertension or hypotensive heart failure, circulatory shock, convulsions, ataxia, fasciculations and coma.

In evaluating envenomation it is important to consider that it is more serious in children and people of advanced age or with debilitating diseases.[35-38]

C. SPECIFIC TOXIN EFFECTS AND COMPLICATIONS
Lachrymation, mydriasis, photophobia, nystagmus, nasal secretion, a characteristic sensation of hair in the throat with dysphonia, cough and dysphagia are some of the most consistent symptoms observed following envenomation.

1. CARDIOVASCULAR
There is ample experimental and clinical evidence that venoms from different scorpion species release catecholamines from the sympathetic nervous system and stimulate the cardiac adrenergic endings. [39-45]
Usually, there is an initial period of hypertension that can be followed by hypotension. Likewise, tachycardia predominates although bradycardia may also be observed. These contradictory effects may depend on the system preferentially affected by the toxins, either cholinergic or adrenergic. [46,47]

The evolution of these phenomena plus the direct action on the conducting system of the heart can lead to cardiac arrhythmia and heart failure and secondarily may cause pulmonary oedema.

Gonzalez-Romero[48] treated 722 patients stung by *Centruroides suffusus*: 38% had electrocardiographic abnormalities, from which 12.8% corresponded to bundle branch block, 10.2% first-degree atrioventricular block and 15% alterations in the ventricular repolarization (in the majority of cases reversible), and the remaining 62% of patients had other abnormalities.

The profuse loss of fluids (sweating, vomiting, diarrhoea) may also contribute to circulatory collapse. Continuous electrocardiogram (ECG) monitoring will permit the early diagnosis and treatment of cardiac arrhythmia and comencement of ventilatory support through oxygen therapy or positive pressure respiration.

2. RESPIRATORY

Tachypnoea is often present. Dyspnoea is a common finding in severe cases. Respiratory failure, the usual cause of death, reflects paralysis of the respiratory muscles, particularly the diaphragm, due to a reflex stimulation of vagal afferent fibers. Bronchial hypersecretion and pulmonary oedema may also be contributing factors.[34,47,49-50] Catecholamines in very large doses may cause brief periods of apnea. Thus, it seems that the pathogenesis of respiratory failure is multifactorial.[50] Respiratory arrest has been occasionally observed following *Centruroides sculpturatus* sting.[38] Most of the available pathological data are based on experimental envenomation. These studies emphasized the presence of different degrees of pulmonary oedema with diffuse parenchymal hemorrhages.[50]

3. NEUROLOGIC

Central nervous system:

There is currently no experimental evidence that scorpion neurotoxins can cross the blood-brain barrier. However, some effects may indicate a direct action on the central nervous system, such as the hyper-excitability and the restlessness, very often observed even in mild cases.[51] The high levels of catecholamines released by the venom may also provide some explanation for these symptoms. Coma can be present in severe envenomation, mostly in children.

Peripheral nervous system

The peripheral nervous system is the main target of scorpion neurotoxins. Most of the time, their clinical effects reflect the action of neurotransmitters, like acetylcholine and catecholamines that are released by the action of the toxins.[51-52]

Autonomic nervous system:

The autonomic nervous system includes parasympathetic and sympathetic pathways. Both are stimulated by scorpion venom. The most common parasympathetic manifestations are: salivation, lachrymation, gastric hyperdistention, diarrhoea, bradycardia and hypotension. The main sympathetic effects are mydriasis, tachypnea, tachycardia and hypertension. In the course of the envenomation one effect can be predominant, but mixed effects are also observed. This is the reason for finding variable symptomatology as discussed by Freire-Maia.[46]

Skeletal and smooth muscle

Spasms, muscle contraction and twitches are due to stimulation of skeletal muscle by the venom.[53] These effects reflect the action of the toxins at a presynaptic level.[54] The most notorious effects on smooth muscle are abdominal pain and diarrhoea. The contraction and or relaxation of smooth muscle of the intestine induced by the venom are due to the release of chemical mediators, such as acetylcholine and catecholamines; but other mediators such as substance P could also be released.[46]

4. GASTROINTESTINAL

Excessive salivation, nausea and vomiting are characteristic features of scorpion envenomation. Also, an increase in motility of the intestine with diarrhoea is common. Gastric hyperdistention is frequently observed. These effects are basically explained by the peripheral action of the toxins on cholinergic nerve fibers (vagus nerve) which would act through muscarinic and H2 receptors.[55-58] Experimental data show a dramatic increase in volume, acid and pepsin output of gastric juice and a significant decrease in its pH. From a clinical point of view it is necessary to be very cautious with patients that suffer peptic ulcers. Acute pancreatitis was reported in humans stung by the scorpion *Tityus trinitatis*

and by *Leiurus quinquestriatus*.[59-62] It was suggested that this could be explained in part by the release of acetylcholine from pancreatic nerve endings.[63] Fletcher *et al.*[64] proposed a mechanism that involves ion channels (Na^+, Ca^{2+} and K^+) in the case of *Tityus serrulatus* venom.

5. MISCELLANEOUS

Skin contact: No effect on intact skin.

Hepatic: The liberated chemical mediators such as catecholamines may provoke increase of liver glycogenolysis, with a consequent hyperglycemic effect in blood.[32,65]

Urinary tract: In severe envenomation urinary incontinence may be observed or urinary retention.[35]

Kidney: *Tityus serrulatus* decreased renal plasma flow, urine and sodium excretion in dogs.[66] These effects could be due to the release of catecholamines from renal nerve endings.

Endocrine and reproductive system: No known effects.

Hematologic: Longnecker reported that the venom of *C.sculpturatus* caused a sustained platelet aggregation in dogs.[67] One possible explanation is that the release of catecholamines induce platelet aggregation and this contributes to a defibrination syndrome. However, the venom of *C. limpidus limpidus* does not seem to affect platelet aggregation in human blood (authors unpublished observations).

Immunologic: In very rare cases of repetitive exposure, allergic reactions have been reported (authors unpublished observations).[68]

Metabolic: A hyperglycemic effect has been documented in *Centruroides* envenomation. The release of catecholamines is expected to increase hepatic glycogenolysis (as mentioned above) and at the same time inhibit insulin release.[69,70]

Acid base disturbances: Blood acidosis associated with hypercapnia occurs in patients with severe respiratory failure, or shock.[50]

Fluid and electrolyte disturbances: Dehydration due to vomiting, profuse sweating and diarrhea must be carefully monitored.

Pregnancy: A case of a pregnant woman (first trimester) who aborted was reported following a sting of *Leiurus quinquestriatus*.[71] There is no information on *Centruroides* species in this respect, nor is there any information on possible foetal damage. However, the toxins can potentiate the action of agonists such as acetylcholine, serotonin and bradykinin enhancing the autorhythmic activity of the uterus.[51]

V. PROPHYLAXIS

A. CIRCUMSTANCES OF STINGS AND HEALTH EDUCATION

In areas endemic for dangerous scorpions it is suggested that shoes and clothes be shaken out before dressing. Since scorpions are nocturnal animals, before going to sleep it is recommended to look between covers, around the bed, walls and ceilings. There are some architectural features of housing that are recommended in order to avoid the entrance of scorpions into houses. The scorpions can not climb smooth and well-polished surfaces. Mazzotti[6] recommends the use of screens on windows and a row of glazed tiles around the outside walls of the house (including stairs) in order to protect against scorpion invasion. The children's bed can be protected by a gauge-cloth envelope.

Accidents occur with both dangerous and non-dangerous scorpions, either in rural or in urban areas. Many accidents take place in houses, buildings or schools. Some species of *Centruroides* invade human dwellings, where they can get into shoes, clothing or inside

furniture. They crawl on walls and ceilings, from where they can fall and sting people. Frequently people are stung while picking up domestic objects containing the hidden scorpions.[33]

In endemic areas, particularly in the dark, it is recommended not to touch unseen objects, but use artificial light.

Additionally, it should be noted that most venomous species of *Centruroides* are resistant to commonly used home-insecticides.[72]

B. PERSPECTIVES ON PRODUCTION OF A VACCINE

In the past, some attempts have been made to prepare a possible immunogen for vaccination of experimental animals.[73] A toxic fraction of the venom from *Centruroides noxius* was detoxified with glutaraldehyde and was shown to produce neutralizing antibodies in rabbits. Purified antibodies protected mice against the lethal effect of the toxic fraction (number II from Sephadex G-50 gel filtration).[73] Detoxification of scorpion toxins by acetylation was also reported for pure toxins of *Androctonus australis* and *Buthus occitanus tunetanus* by Delori et al.[74] These authors conclude that the use of fraction II from Sephadex G-50 is the most suitable substrate for obtaining a potent acetylated anatoxin that is capable of producing neutralizing antibodies against several venoms of African scorpions. A more recent review on this subject is also available.[75] Heneine *et al.*[76] showed that iodination of fraction T2 from the venom of *Tityus serrulatus* abolishes its lethal capacity, without changing its immunological properties.

More recently, several synthetic peptides were prepared with amino acid sequences corresponding to the primary structure of whole toxins and/or segments of the primary structure of toxins of the scorpion *Centruroides noxius* for immunization purposes.[77] Some peptides are immunogenic and produce neutralizing antibodies against the native toxin, when injected in passive immunization protocols. Unfortunately, other peptides cause hypersensitivity of preimmunized animals, when challenged directly with native toxin. These experiments are in progress, and it is difficult to foresee, at this moment, the possibility of a vaccine against scorpion venom.[78]

Several questions should be addressed before a real vaccine can be obtained and applied in humans. The first question, only partially answered by the publications mentioned above,[78] is the selection of an adequate antigen, i.e. homogeneous, non-toxic, which can be obtained in sufficient amounts with an acceptable grade of quality for human use. The second question is the route of administration and the adjuvant needed, if any. The next important question is to determine the levels of neutralizing-circulating antibodies and the duration of such levels after immunization. A scorpion sting is an acute event. The envenomed organism needs a prompt and efficient response. Further problems could be presented by the need to prepare different antigens, according to different geographical areas of the world, or different species of scorpions living in the same area. Finally, an outbred strain, like humans, are bound to present a wide variety of different responses to the same antigens.

Newer approaches, using cloned toxin-genes [79-82] and recombinant DNA techniques might present an alternative strategy for studying the problem of vaccine preparation against scorpion stings. While these questions are awaiting answers the prospective development of a vaccine is no more than a dream, and serotherapy continues to be the only choice for treatment of scorpionism.

VI. FIRST AID TREATMENT

PRINCIPLES AND RECOMMENDATIONS

Scorpion accidents constitute a significant problem, mainly in children under five years old, and elderly and emaciated or malnourished adults. If not adequately treated, often lethal consequences, can result.

Unfortunately, there is no simple way for a medical doctor and the public to distinguish between a dangerous and a harmless species. Several different species can live in the same location.

The following steps can be used in cases of scorpion sting:

- Immobilize the patient and the affected part;
- Avoid incisions;
- A constriction bandage could be placed close to the site of sting, but the application of tourniquets should be avoided;
- The use of local suction is recommended by some, but remains controversial. It is a technique widely accepted in Mexico, where it appears effective, based on anecdotal evidence. Suction is applied at the site of the sting (oral suction is very effective if immediately applied, and providing there is no damage to the oral mucosa; the venom must be spat out immediately);
- Apply cold packs or immerse the stung area in ice water for the first two hours (intermittently) to help slow absorption;
- Obtain medical attention or transport to hospital;
- If the offending scorpion has been killed, take it with the victim to hospital for identification.

The victim must be kept calm and warm, and given reassurance. Immobilize the affected part in a functional position. Watch for any untoward reaction, and transport the victim to the nearest medical facility as quickly as possible. On admission, local antisepsis must be performed: give analgesics if pain is severe. Keep the patient under observation for at least 4 hours. Tetanus prophylaxis is recommended although not mandatory.

Avoid incisions. The local extraction of venom by suction with the mouth through the orifice made by the stinger is often recommended, but its efficacy is not proved. As little as one microgram of purified toxin is potent enough to cause local anaesthesia in humans with impairment of movements (unpublished personal experience). A few tenths of micrograms of purified material are probably lethal to humans.

VII. CLINICAL MANAGEMENT

A. DIAGNOSIS

A patient describing a well documented history of scorpion sting presents no problem in diagnosis. However, the possibility that the victim could have being stung by a non dangerous scorpion should be taken into consideration. Thus clinical observations are of utmost importance in confirming envenoming. With children under the age of four years old it is common to hear them crying loudly, and it is also very common to miss the offending scorpion. In this case if there is sneezing and nasal pruritus one can suspect a scorpion sting and consequent envenomation.

B. TREATMENT

1. ANTIVENOM PRODUCTION

A recent review by Theakston and Warrell[83], compiled addresses and names of most Institutions around the world that produce scorpion antivenoms, which has been updated for this chapter (see also Table__, Chapter 17). The following countries are reported to be currently producing antivenom against scorpions: Algeria, Brazil, England, Germany, India, Iran, Mexico, Morocco, South Africa, Tunisia, Turkey, U.S.A and Venezuela.[83]

In Mexico, *Centruroides* polyvalent antivenom is produced in horses by injecting with a mixture of venom from the most important species (*C. noxius, C. l. limpidus, C. l. tecomanus*, and *C. suffusus suffusus*). It is enzymatically digested and lyophilized. The antivenom cross-reacts with other *Centruroides* spp. venoms, and protects against all venomous species in Mexico. There are two producers of antivenom against *Centruroides* species:

1.- Gerencia General de Biologicos y Reactivos. Health Ministry. M. Escobedo 20, C.P. 11400 Mexico D.F., Mexico. Suero Antialacran - ampoule with lyophilized immunoglobulins. One ampoule neutralizes 150 LD_{50} in mice tested intraperitoneally.

2.- Laboratorios BIOCLON (before MYN, Zapata, and Grupo Pharma). Calzada de Tlalpan 4687, C.P. 14050 Mexico D.F., Mexico. Suero Antialacran (Alacramyn)® - ampoule with lyophilized immunoglobulins. One ampoule neutralizes 150 LD_{50} in mice tested intraperitoneally. ®Registered Trademark of Bioclon, Mexico.

A third producer of antivenom is located in the USA. The antivenom from Arizona is prepared in goats following immunization with venom of *C. sculpturatus*.[84] The lyophilized product is distributed free throughout the state of Arizona. The mailing address is: Antivenom Production Laboratory, Arizona State University, Tempe, Arizona 85281, USA.

Other producers of antivenom for scorpion stings in Latin America, are:
Instituto Ezequiel Dias, Belo Horizonte - Minas Gerais, Brazil

Centro de Biotecnologia, Facultad de Farmacia Universidad Central, Caracas, Venezuela
The Centro de Biotecnologia in Venezuela prepares an antivenom against *Tityus discrepans* (personal communication Dr. Jeanette Scannone).

2. ANTIVENOM THERAPY

In Mexico specific treatment consists of the administration of *Centruroides* polyvalent antiserum. Its application will depend on the presence of two or more general symptoms listed previously (section IV.B).
The quantity of antivenom to be used is determined by the clinical symptoms and their evolution over time. The lyophilized immunoglobulins contained in the ampoules are dissolved in 5 ml of water for injection. There is always uncertainty in the progression of the envenomation and it is almost impossible to predict the evolution of the symptomatology. Thus, in earlier stages of envenomation, one or two vials are enough to control the symptoms. However, when the patient comes to the hospital in later stages of envenomation it might be necessary to use up to four vials of antivenom. This is valid for both children and adults. In Mexico it is considered advisable to administer an antihistamine (i.e., chlorpheniramine) together with antivenom. Faster neutralizing effects

are obtained if the intravenous route of injection is used. Intramuscular injection of the antivenom is also effective. The use of the antivenom is not recommended in the absence of systemic symptoms of envenomation.

Currently there are no uniform criteria for treatment of scorpion sting envenomation. Several reports are available in the literature.[7-9,37,41,44,57,58,85,86]

In Mexico, from a total of 38,068 cases of envenomation by *Centruroides* scorpions, the application of antivenom (mixed with an antihistamine) was reported in 20,293 cases[8]. Skin tests, or other procedures, were not performed to determine possible hypersensitivities. None of the patients developed immediate allergic reactions. No deaths were recorded. Cases of late development of serum sickness, although possible, were not documented, mainly because the patients did not return to the hospital. The conclusion of this work is that there is no doubt that the administration of antivenom is the most important therapeutic measure in neutralizing the circulating venom.

In Brazil, the group of Freire-Maia *et al.*[44] has reported treatment of 3,860 patients stung by *Tityus serrulatus* with a very high rate of success due to the prompt use of antivenom. The mortality rate was 0.28%. All deaths were in children, principally because of late admission to the hospital (3 or more hours after the accident). The serotherapy consisted of intravenous injection of 20 - 40 ml of antivenom from Butantan Institute, Sao Paulo, Brazil.[57,58]

In Tunisia, specific antivenom is considered the main therapeutic measure for severe cases of envenomation by the scorpions *Androctonus australis* and *Androctonus aeneas*.[85]

In Israel, Gueron and Ovsyshcher[41] reported that antivenom therapy is of great value in cases of severe envenomation by *Leiurus quinquestriatus* or by *Buthotus judaicus* when it is used in early stages of envenomation. Similarly, Hershkovich *et al.*[86], used antivenom in 96% of 53 cases in which it was indicated. The offending scorpion was identified as *Leiurus quinquestriatus*. Allergic reactions occurred in 4 cases, two of which developed symptoms of anaphylaxis. Fortunately, none of these cases were fatal.

3. ROLE OF LABORATORY INVESTIGATIONS

Detection of venom in biological fluids of the patient is very difficult, and is not a common practice. Radiolabeled antibodies or immunoenzymatic assays are being prepared by some laboratories for such purposes, and might find future application in toxicological analysis. In the initial steps of evaluating envenomation, standard blood tests are of very little value, however in complicated cases arterial blood gas, serum glucose, red blood cells and amylase levels can be helpful for clinical treatment, if known.

4. EXAMPLES OF CLINICAL TREATMENT

The clinical cases reported here were taken from the records of the Red Cross in Leon, Guanajuato, Mexico.

Case I

Male, 4 months of age. The baby was in his bed and suddenly began to cry. The mother picked him up and discovered a scorpion behind his head. Ten minutes later he was crying very loudly and had excessive salivation, dyspnea, hyperexcitability, nystagmus, bronchial secretions, tachycardia, respiratory failure, abdominal distension, meteorism, ataxic movements and profuse sweating. He was assessed medically 30 minutes after the sting.

The pulse rate was 170, respiration 34, white cells $7.5x10^9/l$ with 5% eosinophils, glucose 142 (normal 60 - 100 mg/dl) (his last feed was 4 hours before). Treated with intravenous fluids and antivenom (only one vial) plus chlorpheniramine intravenously, symptoms disappeared after 3 hours.

Case 2
Female, 48 years. Patient stung twice on her hand. Within five minutes she felt intense pain, local hyperesthesia, stammer, photophobia, excessive salivation and sensation of hair in the throat. At the hospital her arterial pressure was 160/110, pulse rate 102, respiration 28, and there was ocular redness. Laboratory tests: Glucose 212 mg/dl (normal 60 - 100 mg/dl) with glucosuria. Antivenom was given by intravenous route 30 minutes after the sting. Recovery completed within two hours. The patient was sent to a diabetic clinic to investigate possible diabetes mellitus (later analysis indicated that she was not diabetic).

Case 3
Female, 8 years. Patient stung in her hand 45 minutes earlier. On admission to the hospital she had intense pain at the site of the sting, hyperaesthesia, pruritus, nasal pruritus, nasal secretion, salivary secretion, sensation of hair in the throat, cough, bronchorrhea, nausea and vomiting, abdominal pain, tachycardia and hyperexcitability. Treated with one vial of antivenom intravenously plus chlorpheniramine. Recovery was complete within three hours.
In all the above cases, the offending scorpion was positively identified as *Centruroides infamatus infamatus*.

5. OTHER ISSUES IN TREATMENT

Other medical measurements are:

- Fluid and electrolyte monitoring (appropriate administration of intravenous fluid, when required);
- Support of cardiorespiratory functions by control of blood pressure, monitoring vital signs, resting in Fowler position;
- Aspiration of nasopharyngeal secretions;
- Correction of acid - base balance disturbances;
- Tetanus prophylaxis;
- Treatment of pain with non-central nervous system depressing analgesics, such as acetaminophen.
- Precautions, in order to avoid bronchial aspiration by the patient when vomiting.

Atropine, neostigmine and steroids have been used in the past for treatment of scorpion envenomation, but they have not been proved to be of important clinical value in *Centruroides* and *Tityus* cases. Experimental data showed that atropine might potentiate the hypertensive effect and increase the severity of the pulmonary oedema induced by scorpion toxin in the rat.[43] However, if there is severe bradycardia (secondary to sinus arrest or any kind of atrioventricular block), atropine should be given (0.05 - 0.1 mg/kg). Application of drugs such as barbiturates or narcotics might increase central respiratory depression in scorpion envenomation.

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Anexo 3

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TABLE 1: PARTIAL STATISTICAL DATA OF SCORPION STINGS IN MEXICO

YEAR	GUANAJUATO*	MORELOS	NAYARIT	MORTALITY
1981	6649	n.c.	n.c.	0
1982	8044	n.c.	n.c.	1
1983	7342	n.c.	1759	17
1984	7928	1564	1068	25
1985	7289	2641	1063	8
1986	7989	3042	1206	7
1987	n.c.	3775	1510	1
1988	n.c.	3661	1109	6
1989	n.c.	n.c.	1814	0
TOTAL	45,241	14,683	9,529	65
GLOBAL	69,453			

* Only Red Cross Hospital and one clinic of IMSS (Mexican Institute of Social Security) in Leon, Guanajuato. n.c.= not compiled.

FIGURE CAPTIONS

Fig. 1: Geographical Distribution of Mexican Scorpions Dangerous to Humans
The Mexican States are indicated by numbers, while the scorpion species by various graphic representations (see legend).[5]

Fig. 2: Representative Photos of Mexican Scorpions
In A: adult female of the species *Centruroides limpidus limpidus*; B: adult female of *Centruroides noxius*; C: adult *Vejovis mexicanus*, while D is a young *Hadrurus concolorous*. The two latter ones are non dangerous to man.

Fig. 3: Primary Structure of Scorpion Toxins
These amino acid sequences were taken from the indicated references (13-14), where the abbreviations correspond to: Cn, *C. noxius*; Css, *C. suffusus suffusus*; Clt, *C. limpidus tecomanus*; CsE, *C. sculpturatus*; Cll, *C. limpidus limpidus*, and NTX to noxiustoxin.

Toxin Amino acid sequence of Na^+ channel blockers[13]

1 10 20 30 40 50 60 66

Cn2
KEGYLVDKNTGCKYECLKLGDNDYCLRECKQQGYKGAGGYCYAFACWCTHLYEQAIV
WPLPNKRCS
CssII
KEGYLVSKSTGCKYECLKLGDNDYCLRECKQQYGKSSGGYCYAFACWCTHLYEQAVV
PLPNKTCN
CtI
KEGYLVNHSTGCKYECFKLGDNDYCLRECRQQYGKGAGGYCYAFGCWCTHLYEQAVV
WPLPNKTCS
Cn3
KEGYLVELGTGCKYECFKLGDNDYCLRECKARYGKGAGGYCYAFGCWCTQLYEQAVV
WPLKNKTCR
CsE1
KEGYLVKKSDGCKYDCFWLGKNEHNTCECKAKNQGGSYGYCYAFACWCEGLPESTPTY
PLPNK-CS
CsE2
KEGYLVNKSTGCKYGCLKLGENEGNKCECKAKNQGGSYGYCYAFACWCEGLPESTPTY
LPNK-CSS
CsE3
KEGYLVKKSDGCKYGCLKLGENEGCDTECKAKNQGGSYGYCYAFACWCEGLPESTPTY
LPNKSC-
CsE1KDGYLVEK-
TGCKKTCYKLGENDFCNRECKWKHIGGSYGYCYGFGCYCEGLPDSTQTWPLPNK-CT
Cn1 KDGYLVDA-
KGCKKNCYKLGKNDYCNRECRMKHRGGSYGYCYGFGCYCEGLSDSTPTWPLTNKTC-

Toxin Amino acid sequence of K^+ channel blockers[14]

1 10 20 30 39

Cn NTX TIINVKCTSPKQCSKPCKELYGSSAGAKCMNGKCKCYNN
Cn II-10.2 TFIDVKCGSSKECXP...
Cll II-10.11.4 TVINVKCTSPKQCLLPCKQI...