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7th Symposium: Pan-American Section of the International Society on Toxinology on Animal, Plant and Microbial Toxins

Charlottesville, Virginia, USA
August 4-8, 2001

Sponsors:

International Society on Toxinology
University of Virginia
Department of Microbiology
Beckman Coulter
Invitrogen
Laboratorios Silanes
Department of the Army, USA

7th Symposium: Pan-American Section of the IST on Animal, Plant and Microbial Toxins

August 4-8, 2001
Charlottesville, Virginia

IST
International
Society on
Toxinology

Meeting Chair:

Jay W. Fox, Ph.D.
Director Biomolecular
Research Facility,
University of Virginia,
Charlottesville,
Virginia, USA.

Local Organizing Committee:

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August 04th, 2001

Dear Colleagues:

On behalf of the local Organizing Committee and the Program Committee of the 2001 Pan American Meeting of the International Society on Toxinology I wish to welcome you to Charlottesville and the University of Virginia. I believe we have developed an interesting and broad program that will provide scientific stimulation to all.

I wish to thank all the speakers who have agreed to present at the meeting. I would encourage all participants to take this opportunity to meet the speakers and fellow participants to promote scientific discussions outside of the formal sessions and perhaps develop collaborations for future projects.

This meeting would not be possible without the financial support of the U.S. Army, Silanes Laboratories, Beckman Coulter and Invitrogen. I would encourage the participants to express their thanks to these organizations during the course of the meeting.

I specifically wish to thank my colleagues, Drs. Serrano and Valente who served without complaint as the "local" organizing committee and all of my many friends and colleagues who served on the Program Committee and provided many useful suggestions (and cautions) regarding the meeting.

Of course, many, if not all of you have interacted with Ms. Julie Burns, who has served as the secretariat for the meeting. I certainly wish to recognize and thank her for her efforts in handling many of the details involved in the organization of a meeting such as this.

Best wishes for an interesting, enjoyable and pleasant stay in Charlottesville.

Sincerely,

Jay W. Fox
Chair, 2001 Pan American Meeting

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GENERAL INFORMATION *

Office Space During Meeting

The G-I room in Jordan Hall ground floor is available to speakers for slide previews etc.

Group Photo

Group photo will be taken on Sunday at 1:30 PM in front of the Jordan Hall main entrance.

Tuesday Tours

Sign up for tours at the registration desk. Choices will be taken on a first-come first-served basis. Buses will be leaving from the Jordan Hall main entrance at 1300 Jefferson Park Avenue.

Tour 1: Monticello, leaves promptly at 1:30 PM.

Tour 2: Monticello, leaves promptly at 2:30 PM.

Tour 3: Ash Lawn and Jefferson Vineyards, leaves promptly at 1:30 PM.

*** Subject to changes.**

PROGRAM

Saturday, August 4th 2001

Registration 4:00 PM

Opening Talks 7:00 PM

Julia Prado-Franceschi, Campinas, Brazil

"A Retrospective of Toxinological Research in the Department of Pharmacology at UNICAMP, Campinas, Brazil"

George Miljanich, California, USA

"The Calcium Channel-blocking Conopeptide, Ziconotide: a Potent New Analgesic Drug"

Reception 8:00 PM

Sunday, August 5th 2001

Breakfast 7:30 AM

Session I 8:30 AM
Toxins as Potential Biological Tools
Chairperson: Gina D'Suze

Sergio Lizano-Gonzales, San Jose, Costa Rica

"Protein inhibitors of venom phospholipases A2: New tools for dissecting the structure and function of toxic phospholipases A2"

Cesary Marcinkiewicz, Philadelphia, USA

"Biological Activities of Non-RGD-containing Disintegrins"

Philip Lazarovici, Jerusalem, Israel

"Pardaxins, Neuropharmacological Tools for Studying Neurotransmitter Release"

Break 10:00 AM

Session II 10:30 AM
Toxins as Potential Therapeutic Tools
Chairperson: Paulo Melo

Frank Markland, Los Angeles, USA

"A Snake Venom Homodimeric Disintegrin with Potent Anti-Tumor Activity: Does Structure Matter?"

Bruno Lomonte, San Jose, Costa Rica

"Bactericidal and Cytolyic Activities of Peptides Derived from Snake Venom Class II Phospholipase A_2 Myotoxins"

Steven Aird, Fortaleza, Brazil

"Ophidian Envenomation Strategies and the Role of Purines"

Lunch 12:00 PM

Session III 2:00 PM
Structure-Function Studies on Toxins I
Chairperson: Ana Moura-da-Silva

Maria Lanio, Havana, Cuba

"Sticholysin I and II, Two Pore-Forming Toxins from *Stichodactyla helianthus* Interact with Different Membrane-Mimicking Systems"

Dietrich Mebs, Frankfurt, Germany

"A New Conotoxin Blocking Potassium Channels"

Raghuvir Arni, Sao Jose do Rio Preto, Brazil

"Venom Proteins as Structural Model Systems"

Break 3:30 PM

Session IV 4:00 PM
Structure-Function Studies on Toxins II
Chairperson: Eppie Rael

Jonas Perales, Rio de Janeiro, Brazil

"Natural Proteic Inhibitors of Phospholipase A_2 and Myotoxins: Insights into Structural and Functional Relationships"

Ana Moura-da-Silva, Sao Paulo, Brazil

"Jararhagin: Structural aspects and Involvement in Local Effects of *Bothrops jararaca* Snake Venom"

Mario Palma, Rio Claro, Brazil

"The Natural Combinatorial Chemistry Strategy of the Web-Spiders: A Chemodiversity of Acylpolyaminetoxins"

Silanes Workshop 7:00 PM

"Unresolved Questions About Antivenoms"

1.- "The antivenom crisis in Africa: how can this be solved?" (David Theakston). 15 min.

2.- "Convenience of Fab, $F(ab)_2$ and/or IgG depending on scorpion or snake envenomation" (Rafael Otero). 15 min.

3.- "Reactivity of antivenins with venoms from different species" (Alejandro Alagón). 15 min.

4.- "Unsolved questions on the use of antivenins" (Cassian Bon). 15 min.

5.- "Is the use of recombinant proteins to make antivenoms convenient?" (Baltazar Becerril). 15 min.

6.- "Future trends" (Lourival Possani). 15 min.

7.- General Discussion. 30 min.

Gina D´Suze - President Pan American Section
Alejandro Alagón - Secretary Pan American Section
Juan López de Silanes - Laboratorios Silanes

Sponsored by Laboratorios Silanes C.A de C.V. México

Monday, August 6th 2001

Breakfast 7:30 AM

Session V 8:30 AM
Proteomics, Genomics and New Technologies
Chairperson: Gilberto Domont

Andre Menez, Paris, France

"An Experimentally-based Model of the 3D Structure of a Toxin-potassium Channel Complex"

Wagner Fontes, Brasilia, Brazil

"A Proteomic Approach to Toxinology"

Ray Norton, Parkville, Australia

"Toxin Structure and Function in the Genomics Era"

Break 10:00 AM

Session VI 10:30 AM
Scorpion and Insect Toxins
Chairperson: José Gutiérrez

Lourival Possani, Cuernavaca, Mexico

"Novel Classes of Toxic Peptides in the Venom of Scorpions: Structure and Function"

Gina D'Suze, Caracas, Venezuela

"Venom Concentration, IL6, IL1-alfa, TNF-alfa, PTT, PT, Amylase and Glycemia Following *Tityus* Scorpion Sting"

Ana Chudzinski-Tavassi, Sao Paulo, Brazil

"Hemostatic Disturbances and Characterization of a Procoagulant Component from *Lonomia obliqua* Caterpillar Venom"

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Session VII 2:00 PM
Aquatic and Amphibian Toxins I
Chairperson: Angel Yanagihara

William Kem, Gainesville, USA

"Molecular Pharmacology of DMXBA, an Anabaseine Derivative, and its Three Primary Metabolites"

Carlos Sevcik, Caracas, Venezuela

"Identifcation of *Enterobacter* Bacteria As Saxitoxin Producters in Cattles Rumen and Surface Water from Venezuelan Plains"

John Daly, Bethesda, USA

"Biologically Active Substances of Amphibian Skin: Synthesis or Sequestration?"

Break 3:30 PM

Session VIII 4:00 PM
Aquatic and Amphibian Toxins II
Chairperson: Maria Lanio

Angel Yanagihara, Honolulu, Hawaii

"Complex Integrated Pathophysiology of *Carybdea alata* Toxins"

Jose Freitas, Sao Paulo, Brazil

"Peptide Toxins from the Venomous Mollusk *Conus regius* from the Fernando de Noronha Archipelago, PE, Brazil"

Carlos Alvarez, Havana, Cuba

"The Presence of Lipid Favoring a Non-lamellar Phase Could Help the Formation of Sticholysin I and II Pore in the Bilayer Inducing a Toroidal Lipid Pore"

Poster Presentations 8:00 PM

Tuesday, August 7th 2001

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Leonard Smith, Fort Detrick, USA

" Protein Engineering and the Evolution of a Vaccine"

Rafael Otero, Medellin, Colombia

"Severe Bothropic Envenomation: Current Clinical, Epidemiological and Therapeutic Aspects in Colombia"

Joseph Burnett, Baltimore, USA

"*Linuche's* Seabather's Eruption is an Excellent Clinical Teaching Model"

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Session X 10:30 AM
Clinical Aspects and Treatments II
Chairperson: Adolfo de Roodt

Jay W. Fox, Charlottesville, USA

"Jararhagin Functions as a Collagen Agonist on Fibroblast Gene Expression"

Alejandro Alagon, Cuernavaca, Mexico

"Envenomation by Venomous Animals in Mexico: Epidemiological, Clinical and Therapeutical Status"

Cassian Bon, Paris, France

"Effects of Immunotherapy on Venom Pharmacokinetics"

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Outing 1:30 PM

Choice of Tours: Monticello or Jefferson Vineyards and Ash Lawn

Barbecue 6:30 PM
Colonnade Club, University of Virginia

Wednesday, August 8th 2001

Breakfast 7:30 AM

Session XI 8:30 AM
Toxins Affecting the Haemostatic System
Chairperson: Mary Ann McLane

Aura Kamiguti. Liverpool, U.K.

"Toxin-induced Haemostatic Disorders: from Coagulopathy to Antithrombotic Therapy"

Manjunatha Kini, Singapore

"Structure-Function Relationships of Trocarin, a Prothrombin Activator from *Tropidechis carinatus* Snake Venom"

Jose Gutierrez, San Jose, Costa Rica

"Role of Neutrophil Leukocytes in Local Tissue Damage and Skeletal Muscle Regeneration after Injection of the Venoms of *Bothrops asper* and *Bothrops jararaca*"

Break 10:00 AM

Session XII 10:30 AM
Microbial and Plant Toxins
Chairperson: Julia Prado-Franceschi

Demetrius Tsernoglou, Rome, Italy

"Structural Requirements of *Endo*polygalacturonase for the Interaction with PGIP (Polygalacturonase Inhibiting Protein)"

Celia Carlini, Porto Alegre, Brazil

"Plant Proteins with Insecticidal Activity: A New Physiological Role for Canatoxin and Ureases?"

Bradley Stiles, Frederick, USA

"*Clostridium perfingens* Iota Toxin: the Unveiling of a Binary Beauty"

Lunch and Departure

TALK

ABSTRACTS

OPHIDIAN ENVENOMATION STRATEGIES AND THE ROLE OF PURINES.

Steven D. Aird.

Laboratório de Toxinas Naturais, Universidade Estadual do Ceará, Avenida Paranjana, 1700, Itaperí, Fortaleza, CE, 60740-000 BRASIL.

Snake envenomation employs three well integrated strategies: prey immobilization via hypotension, prey immobilization via paralysis, and prey digestion. Purines (adenosine, guanosine and inosine) evidently play a central role in the envenomation strategies of most advanced snakes. Purines constitute the perfect multifunctional toxins, participating simultaneously in all three envenomation strategies. Because they are endogenous regulatory compounds in all vertebrates, it is impossible for any prey organism to develop resistance to them. Purine generation from endogenous precursors in the prey explains the presence of many hitherto unexplained enzyme activities in snake venoms: 5'-nucleotidase, endonucleases (including ribonuclease), phosphodiesterase, ATPase, ADPase, phospho-monoesterase, and NADase. Phospholipases A_2, cytotoxins, myotoxins, and heparinase also participate in purine liberation, in addition to their better known functions. Adenosine contributes to prey immobilization by activation of neuronal adenosine A_1 receptors, suppressing acetylcholine release from motor neurons and excitatory neurotransmitters from central sites. It also exacerbates venom-induced hypotension by activating A_2 receptors in the vasculature. Adenosine and inosine both activate mast cell A_3 receptors, liberating vasoactive substances and increasing vascular permeability. Guanosine probably contributes to hypotension, by augmenting vascular endothelial cGMP levels via an unknown mechanism. Novel functions are suggested for toxins that act upon blood coagulation factors, including nitric oxide production, using the prey's carboxypeptidases. Leucine aminopeptidase may link venom hemorrhagic metalloproteases and endogenous chymotrypsin-like proteases with venom L-amino acid oxidase (LAO), accelerating the latter. The primary function of LAO is probably to promote prey hypotension by activating soluble guanylate cyclase in the presence of superoxide dismutase. LAO's apoptotic activity, too slow to be relevant to prey capture, is undoubtedly secondary and probably serves principally a digestive function. It is concluded that the principal function of L-type Ca^{2+} channel antagonists and muscarinic toxins, in *Dendroaspis* venoms, and acetylcholinesterase in other elapid venoms, is to promote hypotension. Venom dipeptidyl peptidase IV-like enzymes probably also contribute to hypotension by destroying vasoconstrictive peptides such as Peptide YY, neuropeptide Y and substance P. Purines apparently bind to other toxins which then serve as molecular chaperones to deposit the bound purines at specific subsets of purine receptors. The assignment of pharmacological activities such as transient neurotransmitter suppression, histamine release and antinociception, to a variety of proteinaceous toxins, is probably erroneous. Such effects are probably due instead to purines bound to these toxins, and/or to free venom purines.

ENVENOMATION BY VENOMOUS ANIMALS IN MEXICO: EPIDEMIOLOGICAL, CLINICAL AND THERAPEUTICAL STATUS.

Alejandro Alagon.

Instituto de Biotecnologia/UNAM. Av. Universidad 2001, Cuernavaca, Mor. 62210.

Venomous animals are a public-health problem of high magnitude. From 1996 to 1998, 558,741 cases of animal stings and bites were recorded; 76.8% of the accidents and 444 deaths were caused by scorpions (1). In the year 2000, the IMSS (Mexican Institute of Social Security), treated 58,494 cases of scorpion stings (2), whereas the Antiscorpion Service of the Red Cross in the city of Leon (State of Guanajuato) reported close to 10,000 cases. During that year, the IMSS also treated 19,313, 3,545 and 644 accidents caused by bees, spiders and snakes, respectively. Mexico is the largest world's consumer of antivenoms with more than 250,000 vials used every year. The immunotherapy is not only highly efficient but also safe. All Mexican antivenoms are made with purified F(ab')2 fragments (from immunized horses); antivenoms contain negligible amounts of aggregates or albumin, and a small proportion of low molecular mass peptides derived mostly from immunoglobulins. The Mexican Pharmacopeia has created the terms "Fabotherapy" and "Fabotherapeutic" to be applied to antivenoms composed by purified enzyme-digested immunoglobulins. This change in terminology has helped to dispel the notion, once prevalent in the medical community, that the use of antivenoms is unsafe and badly tolerated. Two new Fabotherapeutics have been recently developed for Black-Widow spider and coral snake bites: Aracmyn® and Coralmyn®; a third one to be used in cases of massive bee attacks is in progress. In scorpionism, the appearance of any systemic symptoms is the indication for the administration of antivenom (Alacramyn®); by applying this criterium no fatalities were recorded and the hospitalization time was reduced to less than two hours. In ofidism, the aggressive use of snake antivenom (Antivipmyn®) is of paramount importance not only to save lives but also to limit necrotic sequelae.

(1) Weekly Epidemiological Bulletin, Mexican Health Ministry, Week 52, 1998. Published by the Secretaria de Salud, Mexico City.

(2) Annual Epidemiological Bulletin of the IMSS, 2000. Published by the Instituto Mexicano del Seguro Social, Mexico City.

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THE PRESENCE OF LIPID FAVORING A NON-LAMELLAR PHASE COULD HELP THE FORMATION OF STICHOLYSIN I AND II PORE IN THE BILAYER INDUCING A TOROIDAL LIPID PORE.

C. Alvarez[1], D. Martinez[1], M. Dalla Serra[2], C. Potrich[2], M. Tejuca[1], F. Pazos[1], F.Casallanovo[3], S. Schreier[3], M. E. Lanio[1], E.A. Lissi[4] and G. Menestrina[2].

[1] Centro de Estudio de Proteinas, Dept. Bioquimica, Facultad de Biologia, Universidad de La Habana, Cuba.

Sticholysin I and II (St I and St II), two basic cytolysins from the sea anemone *Stichodactyla helianthus*, efficiently permeabilize lipid vesicles by forming pores in their membranes. To better characterize the lipid dependence of the cytolysin membrane interaction, we have evaluated the effect of including different lipids in the vesicle composition. The inclusion of even small proportions of PA into PC/SM LUVs led to a marked increase in calcein release caused by both St I and St II, reaching maximal effect at ~ 5 mol % of PA. Titration of toxin with PC/SM/PA liposomes induced a sensitive increase in protein fluorescence suggesting a more hydrophobic environment for Trp and promoted changes in FAR-UV CD spectra features when compared to PC/SM vesicle addition. Other negatively charged lipids (phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), or cardiolipin (CL)), all at 5 mol %, also elicited an increase in calcein release, the potency being in the order CL ~ PA>>PG ~ PI ~ PS. It was demonstrated that the effect was not mediated by electrostatic interactions between the cytolysin and the negative surface of the vesicles. It is suggested that the insertion of the toxin channel could imply the formation in the bilayer of a non lamellar structure a toroidal pore. In this case, the presence of lipids favoring a non-lamellar phase, in particular PA and CL, strong inducers of negative curvature in the bilayer could help in the formation of the pore. This possibility is confirmed by the fact that the formation of toxin pores strongly promotes the rate of transbilayer movement of lipid molecules, which indicates local disruption of the lamellar structure.

VENOM PROTEINS AS STRUCTURAL MODEL SYSTEMS.

R. K. Arni.

Department of Physics, IBILCE/UNESP, Sao Jose do Rio Preto-SP.

Venoms and saliva are complex mixtures of peptides and proteins such as inhibitors, disintegrins, myotoxins, cardiotoxins, phospholipases and proteases that play key roles in a wide spectrum of important biological processes as diverse as myotoxicity, membrane damage, cell communication, hemostasis, fibrinolysis and blood coagulation.

Interestingly, these venom proteins display high sequence and structural homology with proteins involved in lipid degradation and blood coagulation and serve as ideal structural models helping to provide a better understanding of the stereo specific requirements of these enzymes in biological processes.

We have been involved in the purification and structural characterization of a number of proteins such as cardiotoxins, disintegrins, phospholipases and proteases which play key roles in these processes.

This talk will focus on the structural aspects of these proteins and their complexes with substrates and inhibitors.

Acknowledgements: This project has been supported by generous funds from FAPESP and CNPq (Brazil).

EFFECTS OF IMMUNOTHERAPY ON VENOM PHARMACOKINETICS.

Cassian Bon.

Unité des Venins, Institut Pasteur, 25, rue du Dr Roux, 75724 Paris, France.

The antivenom immunotherapy is the unique specific treatment of snake and scorpion envenomations. Although widely used and medically accepted, but it is still empirically administered and many questions remain unsolved.

In a first step, ELISAs were developed in parallel with clinical grading scales of viper and scorpion envenomations in France and in Tunisia, respectively. In both cases, a good correlation was observed between the venom levels in the blood and the clinical symptoms, providing that the assay is performed soon after the bite or the sting. In a second step, we examined the kinetics of the venom in the case of rabbit envenomations by viper (*Vipera aspis* in France) and by scorpion (*Androctonus australis garzonii* in Tunisia and *Centruroides limpidus limpidus* in Mexico). The toxic components of *V. aspis* venom are high molecular weight proteins (20-200 kDa while the molecular weight of scorpion toxins is lower than 10 kDa. After intramuscular injection of *V. aspis* venom, the venom resorption follows a complex process: it is fast during the first 24 hr then it occurs at a slower rate over the subsequent 72 hr, resulting in a long half-life of elimination (36 hr). On the other hand, the absorption of *A. a. garzonii* toxins is very fast and complete and its half-life of elimination is short (2 hr).

The effect of immunotherapy was then tested, following the kinetics of viper or scorpion venom, before and after the injection of antivenom. It appeared that the detoxification process is explained by a redistribution of the venom from the extravascular compartment to the vascular one, where it is complexed by the antibodies. Intravenous injection is the most effective route for antivenom administration. In the case of *V. aspis* envenomations Fab'_2 are more efficient than Fab, due to their differential pharmacokinetic parameters. These experimental studies constitute a first approach to a scientific basis for the optimization of the immunological treatment of envenomations. They provide also an experimental model to optimize the immunotherapy.

Linuche'S SEABATHER'S ERUPTION IS AN EXCELLENT CLINICAL TEACHING MODEL.

Joseph W Burnett MD[1], Lourdes Segura-Puertas MD[2] and Edgar P Heimer de la Cotera MD[2].

[1]University of Maryland School of Medicine, Department of Dermatology, Baltimore; [2]Universisdad Nacional Autonoma de Mexico, Centro de Neurobiologia Campu-Juriquilla, Queretaro Instituto de Ciencias del Mar y Limnologia, Cancun, Q. Roo.

Seabather's eruption due to *Linuche unguiculata* appears off the eastern Yucatan coast in the spring between late January and June. During this period the schyphistomae strobilate and ephyrae, medusae then juvenile planulae larvae appear in sequentially but overlapping populations as time progresses. Lesions on the swimmers' skin initially show imprints of ephyrae, later reflect the structures of medusae and finally in May or June consist of small papulopustulo vesicles induced by the larvae. Because of their small size, lesions produced by ephyrae and larvae are usually under the swimsuit where they are trapped, but medusae induced lesions can be elsewhere. "Overlapping" morphologies of the eruption can be common. The resulting disorder resulting from any of the three jellyfish stages is similar. The invited cutaneous manifestations represent a toxic reaction to the jellyfish venom. Second attacks resemble the initial episode. Later sequellae such as intermittent persistent urticaria, second eruption without exposure and ophthalmic problems probably result from a hypersensitivity pathogenesis. Serum antibodies predictably form and persist. Sting prevention can be achieved with protective clothing or heavy greasy topical preparations. Nematocyst inhibition occurs with topical vinegar but is not effective because the vast majority of the firing occurred as the bather was in or exiting from the water. Definitive therapy is lacking and "relief of symptoms" procedures are followed. This disorder shows the importance of the life cycle in designing the rash's morphology, the contrast of toxic versus hypersensitivity induced symptoms, the persistence of serum immunoglobulin long after stinging, the effective use of barrier clothing and ointments, the lesser importance of nematocyst arrest measures and unfortunately the lack of good treatment.

PLANT PROTEINS WITH INSECTICIDAL ACTIVITY: A NEW PHYSIOLOGICAL ROLE FOR CANATOXIN AND UREASES ?

Célia R. Carlini.

Depto.Biofísica-IB, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, CEP 91.501-907. e-mail: ccarlini@vortex.ufrgs.br

Plants produce a wide array of substances including proteins, such as lectins, chitinases, enzyme inhibitors, ribosome-inactivating proteins, etc, in response to a multitude of predators and pathogens. Canatoxin (CNTX)-like proteins and urease constitute a novel group of multifunctional proteins with a putative role in plant defense against insect predation and phytopathogenic fungi. CNTX is neurotoxic protein (a dimer of 95 kDa subunits) from jackbeans *(Canavalia ensiformis)*, lethal to mice and rats by intraperitoneal route, but it is inactive if given orally (1). CNTX is also lethal to a group of insects that relay on cathepsin-like enzymes for digestion, while no effect of the toxin was seen in insect displaying trypsin-based digestion (2). CNTX did not show any inhibitory activity toward the main digestive enzymes of susceptible insects nor did it show any lectin, chitinase or chitin-binding properties that would account for its insecticidal activity. When ingested by susceptible insects, the protein is proteolitically "activated" by cathepsin-like enzymes to give a 10 kDa entomotoxic peptide (3). Determination of CNTX's partial aminoacid sequence indicated a high homology with urease found in the same seed. RT-PCR applied to mRNA isolated from *C.ensiformis* tissues confirmed the presence of two genes sharing 86% similarity. Further studies have shown that canatoxin is a Zn/Ni hybrid isoform of urease with about 30% of its ureolytic activity. As described for CNTX, urease also displayed platelet aggregating activity, interaction with glycoconjugates, and entomotoxic effects. Studies with a classical inhibitor of urease, *p*-hydroximercuribenzoate, suggested that at least two distinct domains are involved in the biological properties of the proteins, as it abolished the ureolytic activity of both isoenzymes without interfering in their interaction with glycoconjugates or the effects on platelets. Altogether, the data support the idea that canatoxin and urease belong to a novel group of multifunctional plant proteins related to defense against insect predation.

1. Carlini CR & Guimarães JA, 1981. *Toxicon* 19, 667-676.
2. Carlini CR et al., 1997. *J. Econ. Entomol.* 90, 340-348.
3. Ferreira-daSilva et al., 2000. *Arch.Insect Biochem. Physiol.* 44, 162-171.

Financial support: CNPq-PRONEX-FAPERGS

HEMOSTATIC DISTURBANCES AND CHARACTERIZATION OF A PROCOAGULANT COMPONENT FROM *Lonomia obliqua* CATERPILLAR VENOM.

A. M. Chudzinski-Tavassi[1] , C. V. Reis[1], P. L Ho[2], M. Zannin[3], R. G. Pozner[4], M. A Lazzari[4] and M. Schattner[4].

[1]Laboratory of Biochemistry and Biophysics; [2]Center of Biotechnology – Butantan Institute , SP. [3]Pathology Dep. – Fed. University of Santa Catarina, SC, Brazil, [4]Dep. Thrombosis and Hemostasis, Nat. Acad. of Medicine, CONICET, Argentina.

Accidental contact with *Lonomia* caterpillar bristles induces an acquired hemorrhagic diathesis. Symptoms of envenoming by *Lonomia obliqua* venom consist of local pain, ecchymosis, headache, acute renal failure and hemorrhagic episodes. We evaluated the venom effect on the components of coagulation and fibrinolytic systems in plasma of 106 patients at the moment of hospital admission. Marked prolongation in coagulation times occured with significant reduction of fibrinogen in about 70% of patients. Decrease of factor V, VIII and protein C was also observed. A very significant rise of D-dimer levels occured without alterations of t-PA or u-PA, and a moderate reduction of plasminogen and alpha 2 antiplasmin were observed. These data suggested a consumptiom coagulopathy and a secondary fibrinolysis.

Looking for venom activities, we initially prepared a crude extract from *Lonomia obliqua* bristles which exerted procoagulant activity due to a Factor X, and prothrombin activating activity. Then, a prothrombin activator protease of 69 kDa named Lopap was purified from this extract. *In vivo,* Lopap was able to evoke thrombus formation in microcapillar vessels of rats, fibrinogen depletion, 30% reduction on platelet number and inhibition of platelet aggregation induced by colagen. Histologycal analysis of isolated tissue, after 1h Lopap e.v. injection revealed glomerular infiltration and tubular necrosis. *In vitro*, treatment of HUVECs with Lopap significantly increased ICAM-1 in a concentration dependent manner. vWf synthesis or release was observed after incubation of endothelial cells with different concentrations of Lopap .

The titration of the putative reactive serines of Lopap by NPGB indicated the stoichiometry of 1.2 serine residue per molecule of NPGB. The kinetic hydrolysis parameters of the internally quenched fluorescent peptide substrate, based on prothrombin sequence, Abz-YQTFFNPRTFGSQ-EDDnp, obtained by Lopap activity were K_{mapp} 4.5 μM; k_{cat} 5.32 sec^{-1}; k_{cat}/K_{mapp} 1.2 x 10^6 $M^{-1}.sec^{-1}$. These values indicated good affinity and a high catalytic efficiency .

Total RNA was extracted from the bristles of *L. obliqua* and a cDNA library was constructed in the pGEM11zf(+) plasmid (Promega). Lopap cDNA (900 bp) was amplified from this library by PCR using degenerated primers corresponding to N-terminal sequence of protein. This fragment was sequenced and sub-cloned in *E. coli* expression vector for recombinant protein production. (Supported by CNPq and FAPESP, Brasil and CONICET, Argentina).

BIOLOGICALLY ACTIVE SUBSTANCES OF AMPHIBIAN SKIN: SYNTHESIS OR SEQUESTRATION?

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Amphibian skin has provided a diverse array of biologically active, and in some cases toxic substances, certain of which have proven to be invaluable as research tools. An incredible array of peptides occur over a wide range of amphibian taxa and have apparently evolved either as antibiotics or as vasoactive noxious substances. Some are toxic, some perhaps psychoactive. Toxic proteins also occur, as do a variety of biogenic amines, the latter noxious at the high levels found in certain lineages of amphibians. The toxic bufadienolides, which target sodium-potassium ATPases, occur at high levels only in bufonid toads, and are produced by the toad. Tetrodotoxins occur in only a few lineages of amphibians, where as potent toxins they serve as noxious substances against potential predators. It appears likely that, unlike the peptides, proteins, amines and bufadienolides, tetrodotoxins derive from a symbiotic microorganism. Lipophilic alkaloids represent the last major class of noxious/toxic substances found in amphibian skin. Such alkaloids include the steroidal samandarines, unique to the fire salamander and apparently produced by that amphibian, the batrachotoxins of the true poison-dart frogs and certain passerine birds, probably obtained from a unknown, mysterious dietary source, the pumiliotoxins/allopumiliotoxins/homopumiliotoxins, known to be from dietary arthropods whose identities are as yet unknown, the histrionicotoxins, gephyrotoxins, decahydroquinolines, and various izidines, all known or proposed to be from dietary ants, the spiropyrrolizidine oximes known to be from dietary millipedes, the tricyclic cocinellines from dietary beetles and the potent analgetic epibatidine of unknown origin. The indolic pseudophrynamines, unique to myobatrachid frogs of the genus *Pseudophryne*, are remarkable in being synthesized by the frog, rather than being sequestered from diet as is apparently the case for all of the other lipophilic alkaloids of frog/toad skin, where the evolutionary event appears to have been the overexpression of an alkaloid-sequestering system.

VENOM CONCENTRATION, IL6, IL1-ALFA, TNF-ALFA, PTT, PT, AMYLASE AND GLYCENIA FOLLOWING *Tityus* SCORPION STING.

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A sandwich ELISA was set up for measuring *Tityus* venom levels in plasma of 121 accidentally envenomed humans. These cases were classified as local symptomatology (LS, pain at the sting site) (n=102) or systemic symptomatology (SS): moderate (local pain, vomiting, sialorrhea, tachycardia, arterial pressure disorders) (n=17) or severe (as moderates plus pancreatitis, respiratory distress) (n=1). Concentrations of venom (all data presented as median and its 95% CI and in order of cases' severity) were 0.92 (0.19, 3.9) ng/ml, 9 (0.7, 83) ng/ml and 31.8 ng/ml. Concentrations of IL-6 were: 1.6 (0.3, 6.6) pg/ml, 7.2 (3, 11.7) pg/ml and 28.6 pg/ml. Concentrations of IL1-alfa were : 0.3 (0.05, 0.9) pg/ml, 1 (0.4, 1.7) pg/ml. Eighty percent of the LS cases had TNF-alfa values between 0 and 5 pg/ml and the rest had values between 5 and 60 pg/ml. All SS cases had TNF-alfa values between 0 and 5 pg/ml. The highest values of venom, IL-6 and IL1-alfa concentrations were observed in patients with SS. The highest values for venom concentration, IL1-alfa and TNF-alfa occurred during in the first 2 hours of envenoming. IL6 was also higher during the first 2 hour in LS cases, but it peaked only after 4 hours in SS cases. Prothrombine time (PT) did not change, but the partial thromboplastine time (PTT) was significantly altered in 15% of LS cases and in 19% of SS cases; suggesting that the venom is acting on the intrinsic coagulation pathway. Amylase and glycemia were increased only in SS cases. This work was supported by an IVIC-SILANES grant.

A PROTEOMIC APPROACH TO TOXINOLOGY.

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Toxinology study today

The progress of the toxinology study elucidated mechanisms of action, helped the development of new drugs and brought many new toxins. It also showed the diverse possibilities of this field, making clear that there is a large number of toxins yet to be identified and characterized.

Usual approaches

The discovery of new bioactive compounds from natural extracts is usually initiated by the observation of a biological activity. Most efforts focus on the evaluation of such activity, correlating it to clinical applications and determining the molecule structure and action pathway.

Although the value of the traditional approach is undeniable, a new approach using the proteomics strategy has come to evidence recently in many areas, including toxinology. In this way, a discovery-based methodology is applied to proteic toxins, being the proteins first characterized and then their activity analyzed.

The proteomic advantages and disadvantages

Many problems in toxinology involve protein identification and characterization. The new science of proteomics addresses these several needs by combining 2-D gel electrophoresis with very sensitive methods of polypeptide identification to provide analysis at the protein level, namely, characterization of a complex tissue with respect to the number and identity of the proteins present. The worth of the proteomic approach has been demonstrated in a range of biological systems. For toxins, numerous applications of proteomics include studies of genetic diversity, phylogenetic relationships, mutant characterization and the effects of abiotic stress.

Other successful examples of the application of proteomics on toxinology are the studies of animal venoms, either comparative or aiming to drug discovery and the studies to map receptors on the surface of cells. Also studies based on the comparative analysis of proteomes have been used to characterize toxin effects on complex living systems.

Our experience

At the Brazilian Center for Protein Research and Services – CBSP, located at the University of Brasilia – Brazil, we are developing research lines involving the proteomic analysis of animal venoms. Some of the studies being currently performed include the analysis of the venom from spiders, scorpions, frogs and plants, respectively from the genus *Loxosceles*, *Tityus*, *Odontophrynus* and *Enterolobium*.

JARARHAGIN FUNCTIONS AS A COLLAGEN AGONIST ON FIBROBLAST GENE EXPRESSION.

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The integrins $\alpha 2\beta 1$ and $\alpha 1\beta 1$ have been shown to modulate cellular activities of fibroblasts upon contact with fibrillar collagen. Previous findings have elucidated the role of $\alpha 2\beta 1$ in the regulation of matrix metalloproteinase (MMP)-1 and membrane-type (MT)-1-MMP expression. The snake venom protease jararhagin has been demonstrated to block type I collagen-induced platelet aggregation by binding to the I-domain of $\alpha 2\beta 1$ integrin and cleaving of the $\beta 1$ subunit, thereby inhibiting integrin-mediated intracellular signaling events. Here we present evidence that in contrast to platelets, the integrin receptor $\alpha 2\beta 1$ in fibroblasts is activated by jararhagin. Jararhagin did not influence the collagen-induced up-regulation of MMP-1 and MT1-MMP of fibroblasts grown in collagen gel cultures. However, these MMPs were significantly induced, as observed by their transcript and protein levels, by jararhagin when fibroblasts were grown as monolayers. Inactivation of the metalloproteinase activity of jararhagin did not abolish this induction thereby indicating that the metalloproteinase activity is not a prerequisite for this effect. Thus, the results suggest that in fibroblasts the snake venom metalloproteinase jararhagin functions as a collagen-mimetic substrate which binds to and activates integrins.

PEPTIDE TOXINS FROM THE VENOMOUS MOLLUSK *Conus regius* FROM THE FERNANDO DE NORONHA ARCHIPELAGO, PE, BRAZIL.

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Conus is a genus of carnivorous mollusks that paralyze their prey by injecting a complex mixture of biologically active peptides that interact with receptors and ion channels. As no Brazilian species have been studied so far, and as each species has a wide variety of novel peptides of pharmacological interest, we have initiated a characterization of peptides in the *Conus regius* venom. This abstract will introduce a peptide from a new conotoxin family.

Thirty specimens of *Conus regius* (length of shell = 3.0 to 4.5 cm) were collected in a scuba diving trip to the Fernando de Noronha Archipelago, PE, Brazil. In order to characterize their feeding behavior, the animals were placed in tanks and they were offered fish (*Bathygobius soporator*), fire worms (*Eurythoe complanata*) and other mollusks. *Conus regius* is a worm-hunting snail, as all of them preyed on the fire worms, *Eurythoe complanata*, easily found in its natural habitat.

After these *in vivo* observations, the *Conus regius* venom ducts were dissected and the venom was lyophilized. Lyophilized venom (500 mg) was extracted with 20%, 40% and 60% acetonitrile and the supernatants were combined (crude venom extract). This extract was fractionated on a semipreparative column in HPLC. One of the major peaks was then purified in an analytical column Vydac C_{18}. The peptide was reduced and alkylated for sequencing at the Peptide Core Facility of the University of Utah, Salt Lake City, UT, USA. Its mass spectrum was measure by MALDI-TOF. The amino acid sequence revealed a 44 amino acid peptide (4,688 Da) with four disulfide cross-links, belonging to a new family of conotoxins.

The biological activity of the purified peptide was tested by intracranial injection in 13-15-day-old Swiss Webster mice. The higher the concentration injected, the more sensitive to touch the animals became. The animals retained the hypersensitivity phenotype with a 1-nmol injection after 2 hours, while they recovered with lower concentrations.

The pattern of cysteines (C-C-CC-CC-C-C) with four disulfide cross-links and the excitatory symptomatology characterize a new group of the conotoxins, the "I superfamily" (R. Shetty, F. Abogadie, E. Jimenez, M. Lirazan, J. Rivier, C. Walker, L. Cruz and B. Olivera, unpublished results), under which this *Conus regius* peptide can be classified. According to the nomenclature used (McIntosh *et al.*, 1999), this peptide will be called **rg11a**.

Further research is required in order to characterize the precise site of action of the *Conus regius* I-conotoxin family members.

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ROLE OF NEUTROPHIL LEUKOCYTES IN LOCAL TISSUE DAMAGE AND SKELETAL MUSCLE REGENERATION AFTER INJECTION OF THE VENOMS OF *Bothrops asper* AND *Bothrops jararaca*.

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Envenomations by the snakes *Bothrops asper* and *B. jararaca* induce a complex inflammatory reaction associated with edema, pain and a prominent leukocyte infiltrate, in which neutrophils predominate. Inflammatory cells recruited at the site of venom injection display enhanced phagocytosis and hydrogen peroxide production. These observations prompted the study of the role of neutrophils in the local tissue damage induced by these venoms, and by a myotoxic phospholipase A_2 (myotoxin I) isolated from *B. asper* venom. Male Swiss mice were pretreated with either an anti-mouse neutrophil rat monoclonal IgG antibody or with a control, irrelevant rat IgG. Anti-neutrophil treatment completely depleted neutrophils from peripheral blood, whereas control IgG did not affect neutrophil counts. Then, mice were injected with either *B. asper* or *B. jararaca* venoms, and the extent of hemorrhagic, myotoxic and edema-forming effects was assessed. No significant differences in these local effects were observed between mice pretreated with anti-neutrophil antibodies and those receiving control IgG. Moreover, myotoxicity induced by *B. asper* myotoxin I was similar in neutrophil-depleted and in control mice. The role of neutrophils in the process of skeletal muscle regeneration was also assessed in groups of mice injected i.m. in the gastrocnemius with the venoms and myotoxin. *B. jararaca* venom induced a moderate myotoxic effect and a drastic hemorrhagic activity, whereas *B. asper* venom induced prominent myotoxicity and moderate hemorrhage. Myotoxin I injection provoked widespread myonecrosis without hemorrhage. Muscle regeneration was assessed by quantitating the muscle levels of creatine kinase and by histological analysis. A deficient regenerative process was observed in mice injected with *B. jararaca* and *B. asper* venoms, since after 7 days regenerative cells were intermixed with remnants of necrotic muscle fibers and with fibrotic tissue. Such poor regeneration is probably due to the disruptive effects that these venoms exert in the microvasculature, as an adequate blood supply is a key requisite for regeneration. Mice depleted of neutrophils and then injected with *B. asper* venom showed a lower regenerative response than mice pretreated with control IgG. Moreover, a drastic difference in the regenerative response was observed in mice injected with myotoxin I, since animals pretreated with control IgG showed a successful regeneration, whereas those depleted of neutrophils had abundant areas of necrotic tissue that had not been removed 7 days after injection. It is concluded that (1) neutrophils do not play a direct role in the acute pathological alterations induced by the venoms of *B. asper* and *B. jararaca*, and (2) neutrophils play a protagonic role in the process of skeletal muscle regeneration after *B. asper* venom and myotoxin I injection, probably associated with the phagocytosis of necrotic material and the recruitment other inflammatory cells, e.g. macrophages, two events directly associated with a successful muscle regenerative response. Supported by FAPESP and University of Costa Rica.

TOXIN-INDUCED HAEMOSTATIC DISORDERS: FROM COAGULOPATHY TO ANTITHROMBOTIC THERAPY.

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Toxins from viperid and crotalid snakes can alter systemic haemostasis or the balance between blood coagulation and fibrinolysis, maintained by non-activated clotting factors, platelets and intact blood vessels. This alteration is successfully achieved due to powerful activators/inhibitors present in the toxins. Although many purified toxins have been characterised and their mechanism of action in vitro known, the impact of such components in envenomed cases is not totally predictable. This is mainly attributable either to the complexity of venom composition or to secondary response/release of pharmacologically active endogenous substances by the toxin(s). However, it is generally acknowledged that powerful activators such as the thrombin-like enzymes (TLE) which directly clot fibrinogen, and the activators of factor X or prothrombin, are responsible for poor blood coagulation in the envenomed patients due to consumption coagulopathy. In contrast, the effects of platelet-aggregating/ inhibiting agents present in the whole venom (lectin types, disintegrins, phospholipases etc) may not be so obvious in vivo. Nevertheless, venom-derived products have been proved useful as tools in the understanding of the blood coagulation mechanism in the past, and more recently in investigation of pathways underlining platelet activation. Platelet/clotting activation within altered blood vessels can lead to thrombotic disorders (stroke and heart attack, two of the major killers in the western world). Because some toxins have specific targets within the haemostatic system, their potential as antithrombotic drugs capable of preventing such disorders awaits to be explored in the future.

MOLECULAR PHARMACOLOGY OF DMXBA, AN ANABASEINE DERIVATIVE, AND ITS THREE PRIMARY METABOLITES.

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DMXBA (GTS-21), an Alzheimer's and schizophrenia drug candidate which is a benzylidene derivative of anabaseine, a nemertine toxin, selectively stimulates alpha7 nicotinic receptors. It rapidly enters the brain after oral administration and enhances cognitive behavior. Less than 1% of orally administered DMXBA is recovered in the urine. This paper reports the identification and characterization of the initial metabolites of this drug candidate. Using rat liver microsomes, we show that the hydroxy metabolites are generated by O-dealkylation of the two methoxy substituents. The compounds also occur in the plasma of rats orally administered DMXBA. All three hydroxy metabolites are partial agonists upon human and rat alpha7 receptors expressed in the *Xenopus* oocyte. The 4-OH and 2-OH monohydroxy metabolites, based on their ionized concentrations, respectively display 7- and 3-fold higher affinities than DMXBA in displacing iodinated BTX binding to rat brain alpha7 receptors. For the rat brain alpha4-beta2 receptor, the affinities of these two metabolites are respectively 3-fold higher and 2-fold lower than for DMXBA. Each metabolite displays a similar efficacy for stimulating rat and human forms of the alpha7 receptor. Predicted metabolite conformations are nearly identical with that of DMXBA. The hydroxy metabolites were predicted and found to attain much lower concentrations in the rat brain than DMXBA. Thus, while they display more potentin vitro effects on the alpha7 receptor, their direct contribution to the effects of orally administered DMXBA may be limited. New methods of delivery across the blood:brain barrier are being explored.

STRUCTURE-FUNCTION RELATIONSHIPS OF TROCARIN, A PROTHROMBIN ACTIVATOR FROM *Tropidechis carinatus* SNAKE VENOM.

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Snake venom prothrombin activators have been classified into four groups based on their structure and cofactor requirements. Group D activators are two-chain serine proteinases and require factor Va, calcium and phospholipids for optimal activity. Thus, they resemble coagulation factor Xa in their functional properties and their structural studies should provide us insight into prothrombinase complex formation. We recently determined the complete amino acid sequence of trocarin, a prothrombin activator from *Tropidechis carinatus* (rough scaled snake) venom. Trocarin shows highly homology (~70%) and similar domain structure to factor Xa and is the first procoagulant protein that is structurally and functionally similar to coagulation factor. Here, we present structure-function relationships of trocarin based on a detailed structural comparison of trocarin with factor Xa. We will discuss its two physiologically important interactions with factor Va and effector proteinase receptor 1 (EPR1), a receptor that plays a critical role in non-hemostatic functions of factor Xa.

STICHOLYSIN I AND II, TWO PORE-FORMING TOXINS FROM *Stichodactyla helianthus* INTERACT WITH DIFFERENT MEMBRANE-MIMICKING SYSTEMS.

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Sticholysins I and II are two basic pore-forming polypeptides purified from the Caribbean Sea anemone *Stichodactyla helianthus* showing a close similarity in terms of primary, secondary and tertiary structure and exhibiting only minor differences between them. They insert into cell membranes and induce a colloid-osmotic imbalance due to the uncontrollable entry of water into the cell through a ~ 2.0 nm diameter pore provoking its death. Experimental evidences suggest that these proteins form an oligomeric structure in the lipid bilayer comprising 4 monomers of the toxin. Accordingly, structural predictions for St I using the INSIGHT/HOMOLOGY MSI software showed a tetrameric oligomer in the membrane stabilized by complementary surface electrostatic interactions among monomers and with a TM sequence constituted approximately by 13 residues. EPR and CD spectra according to fluorescence data showed that both toxins interact with lipid bilayers. Furthermore, binding experiments demonstrated that more than 90% of St II was inserted into liposome membranes independently of the bilayer physical state. However, the toxin was associated with the lipid bilayer, at least in two ways as a function of the membrane physical state, irreversibly and reversibly bound, this latter probably with a non-defined organization and easily extracted by high affinity membranes. In addition, noticeable changes in CD and fluorescence spectra properties of both toxins were also observed in the presence of other microheterogeneous systems like surfactants that could be correlated to variation of their hemolytic activity indicating the protein association with these systems.

PARDAXINS, NEUROPHARMACOLOGICAL TOOLS FOR STUDYING NEUROTRANSMITTER RELEASE.

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Pardaxins, a family of polypeptide, excitatory neurotoxins with pore-forming activity were isolated from the gland secretion of *Pardachirus* sp. fish and used as pharmacological tools to investigate mechanisms of neurotransmitter release from neurons. In pheochromocytoma PC12 cells, a neuronal model to study exocytosis, Pardaxins form voltage-dependent pores which induce increase in intracellular calcium and calcium-dependent dopamine and ATP release. Pardaxins stimulate with different kinetics the extracellular signal-regulated protein kinases (MAPKs) super family of enzymes. Pardaxin stimulated MAPK phosphorylation activity within 15 min and p38 and JNK activity at 30 and 120 min, respectively. We propose that MAPK activation is involved in Pardaxin induced neurotransmitter release while the delayed activation of stress-kinases p38 and JNK is related to pardaxin-induced neurotoxicity. Pardaxins stimulate the arachidonic acid cascade generating arachidonic acid and eicosanoids. Pardaxins-induced dopamine release was correlated to stimulation of both calcium-dependent, cytosolic ($cPLA_2$) as well as calcium-independent ($iPLA_2$) phospholipases A_2. Pardaxins represent novel pharmacological tools to study the signal transduction of synaptic transmission.

PROTEIN INHIBITORS OF VENOM PHOSPHOLIPASES A_2: NEW TOOLS FOR DISSECTING THE STRUCTURE AND FUNCTION OF TOXIC PHOSPHOLIPASES A_2.

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Within the last decade, a number of proteins naturally present in the blood of snakes have been found to possess inhibitory properties towards the enzymatic and toxic properties of phospholipases A_2 (PLA_2s) present in snake venom and other sources. These inhibitors have been classified into three groups: α-type inhibitors which possess carbohydrate recognition domain (CRD)-type motifs; β-type inhibitors which contain leucine repeats reminiscent of a human albumin-type plasma protein; and γ-type receptors which contain three-finger domains. All three inhibitors have been isolated and/or cloned from snakes of the crotalid and viperid subfamilies, while the α-type and γ-type inhibitors have also been found in the colubrid and elapid families, respectively. These inhibitors block PLA_2 activity and/or toxicity by forming stable complexes with their target PLA_2s. The α-type inhibitors bind to and neutralize class II PLA_2s, whereas the γ-type inhibitors neutralize all three classes (I, II, and III) PLA_2s found in snake and bee venoms, as well as other non-venom sources. Studies in our laboratory have resulted in the characterization of the α- and γ-type inhibitors from Central American crotalid snakes. Binding and neutralization analysis with the α-type inhibitors from *Bothrops asper* (BaMIP) and *Cerrophidion godmani* (CgMIP-II) further demonstrate a selectivity towards inhibition of basic class II PLA_2s. These PLA_2s are further subdivided into the enzymatically active Asp^{49} variant as well as the Lys^{49} non-active variant, both of which are myotoxic. Both types of myotoxins from the venom of *B. asper* are inhibited by BaMIP and CgMIP-II. However, these inhibitors only neutralize the Lys^{49} variant from the *C. godmani* venom, and not the Asp^{49} isoform, which is rather inhibited by the γ-type inhibitor. Recent determination of the amino acid sequence of the Asp^{49} myotoxic PLA_2 from *C. godmani* revealed important sequence divergence from the other Asp^{49} and Lys^{49} isoforms from both snakes and seems to be unique among currently determined viperid myotoxins. These results suggest important structural differences among class II PLA_2s which possess similar toxic properties, the structure and function of which can be addressed through protein inhibitors as tools for selective binding and biological neutralization studies.

BACTERICIDAL AND CYTOLYTIC ACTIVITIES OF PEPTIDES DERIVED FROM SNAKE VENOM CLASS II PHOSPHOLIPASE A_2 MYOTOXINS.

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The basic class II phospholipases A_2 (PLA_2s) present in the venoms of crotalid snakes constitute a family of 15 kDa proteins which exert skeletal muscle-damaging activity at the site of injection. Both the "Asp49" and "Lys49" isozymes of this family possess a conserved three-dimensional architecture and common toxic/pharmacological activities, but differ strikingly in their ability to perform catalysis, as only the former are enzymatically active. The study of some Lys49 proteins has revealed the existence of a cationic-hydrophobic segment near their C-terminus that can reproduce (albeit with a lower potency) several of the toxic actions of the whole molecule. In the case of *Bothrops asper* (Lys49) myotoxin II, the 13-mer peptide 115-129 (KKYRYYLKPLCKK) induces cytolysis in vitro, and reproduces its bactericidal effect against several Gram-negative and Gram-positive bacteria. In the case of a Lys49 PLA_2 from *Agkistrodon p. piscivorus*, the corresponding 13-mer peptide (KKYKAYFKLKCKK) is cytolytic, bactericidal, and causes myonecrosis in mice. Thus, region 115-129 of these two Lys49 PLA_2s is clearly involved in the mechanism of membrane damage responsible for their myotoxic action. On the other hand, the observation that short synthetic peptides can reproduce the bactericidal action of the proteins, has raised interest in their potential as novel antibiotics. Peptide 115-129 of *B. asper* myotoxin II is much more toxic to bacteria than to eukarionic cells, being able to kill medically relevant strains of *Salmonella*, *Pseudomonas*, *Shigella*, *Vibrio cholerae*, or *Staphylococcus aureus*, for example. This peptide rapidly permeabilizes bacterial membranes to fluorescent probes, in similarity with its parent PLA_2 protein, myotoxin II. The reported activity of this toxin and its 13-mer peptide demonstrated for the first time a bactericidal mechanism that is independent of a PLA_2 catalytic activity in this group of enzymes. Recent evidence suggests that peptide 115-129 does not act through the recognition of a proteic target structure. Variants of peptide 115-129 with an enhanced bactericidal activity have been obtained. However, a major limitation of their possible use is the concomitant increase in toxicity towards mammalian cells. The observation that snake venom myotoxic PLA_2s, and selected peptide fragments, display a potent antimicrobial activity, further exemplifies the vast pharmacological potential of natural products from the tropical biodiversity.

BIOLOGICAL ACTIVITIES OF NON-RGD-CONTAINING DISINTEGRINS.

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Disintegrins are low molecular proteins isolated from many species of vipers. They can be divided into two major groups: monomeric and dimeric disintegrins. Most of monomeric disintegrins contain RGD sequence in their active site and are potent inhibitors of fibrinogen receptor, an alfaIIbbeta3 integrin. Dimeric disintegrins may appear as homo- or hetero-dimers. Heterodimeric disintegrins show sequence variability in their active site. Except having RGD motif, they may contain MGD (EMF10), MLD (EC3, EC6, VLO5), VGD (EC3, VLO5), WGD (CC8). Presence of MGD motif in EMF10 increased potency and selectivity of this disintegrin to inhibit alfa5beta1 integrin. Disintegrins containing MLD motif are potent inhibitors of leukocyte integrins alfa4beta1, alfa4beta7 and alfa9beta1. They potently block binding of alfa4beta1 integrin to VCAM-1 and alfa4beta7 to MadCAM-1. Moreover, EC3 and EC6 inhibit binding of alfa9beta1 integrin to VCAM-1 but have no effect on binding this integrin to other ligands such as tenascin-C and osteopontin. The WGD sequence present in B subunit of CC8 increases activity of this disintegrin to inhibit RGD-dependent integrins such as alfaIIbbeta3, alfavbeta3 and alfa5beta1. By peptide synthesis it has been found that WGD sequence may mimic RGD motif. Moreover, peptides containing WGD sequence were much more active than RGD-peptides in inhibition of three above integrins. Most recently, the new short, monomeric disintegrin has been isolated from venom of *Vipera lebetina obtusa*. This disintegrin called obtustatin, has identical pattern of cysteines like other short, monomeric disintegrins, echistatin and eristostatin. However, obtustatin does not contain RGD sequence and does not show any inhibitory activity characteristic for RGD-containing disintegrins. Obtustatin is potent and selective inhibitor of alfa1beta1 integrin, which is specific receptor for collagen type IV. By a short peptide synthesis the KTS sequence has been found as an active site of obtustatin.

A SNAKE VENOM HOMODIMERIC DISINTEGRIN WITH POTENT ANTI-TUMOR ACTIVITY: DOES STRUCTURE MATTER?

Frank Markland.

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Contortrostatin (CN) is a 13.5 kDa disintegrin isolated from southern copperhead snake venom. Similar to other disintegrins, CN contains an RGD motif that can be used to target integrins. However, CN is structurally unique in that it is a homodimer. We have shown that CN has impressive anti-tumor activity in animal models of human breast and ovarian cancer. For the ovarian cancer studies, we used OVCAR-5, a human epithelial carcinoma cell line of the ovaries. We utilized a xenograft model in which cultured OVCAR-5 cells, which are essentially devoid of integrin $\alpha v\beta 3$ but are $\alpha v\beta 5$ positive, are injected intraperitoneally (i.p.). Metastasis in this model closely mimics that found in humans. We used i.p. injection of CN to investigate its anti-tumor activity. Tumor dissemination was studied by gross examination and survival rates. The anti-angiogenic potential in CN-treated versus control groups was studied via factor VIII immunohistochemistry and image analysis. CN treatment was begun 1 week after introduction of 10^6 tumor cells. A bi-daily CN treatment regimen (20 μg per injection) was employed. The overall tumor burden in some of the CN-treated mice was so greatly decreased as to not provide any tumor sites for angiogenic study. The results of this study showed that CN can be injected into the peritoneal cavity of an animal model without detectable side effects. CN was able to inhibit secondary sites of metastasis and prevent angiogenesis. In separate studies, we found that treatment of integrin $\alpha v\beta 3$ positive breast cancer cells with CN induces integrin-mediated tyrosine phosphorylation events and causes severe disruptions in the actin cytoskeleton and disassembly of focal adhesion structures. These events are mediated exclusively by the $\alpha v\beta 3$ integrin and are likely the result of CN-mediated cross-linking of this receptor at the cell surface, since monovalent disintegrins, flavoridin or echistatin do not induce such effects. The results suggest a novel integrin-mediated mechanism by which cell motility can be inhibited and suggest an alternative approach to therapeutic intervention for cancer invasion and metastasis. However in the predominantly $\alpha v\beta 3$ negative OVCAR-5 model, this mechanism presumably does not come into play.

A NEW CONOTOXIN BLOCKING POTASSIUM CHANNELS.

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From extracts of the venom glands from the marine snail *Conus virgo* a novel peptide-toxin, named ViTx, has been isolated by gel filtration and HPLC. It consists of 35 amino acid residues and contains four disulfide bonds. The amino acid sequence was partially determined by Edman degradation. The complete sequence was established by molecular biological analysis of gene fragments from cDNA by using primers designed according to the partial structure of the toxin. Voltage-clamp studies on various ion-channels expressed in *Xenopus* oocytes indicate that the toxin is a specific blocker of potassium channels, particularly of the Kv 1.1 and Kv 1.3 type. ViTx is the first toxin targetting vertebrate potassium-channels. The amino acid sequence of the toxin (SRCFPPGIYCTSYLPCCWGICCSTCRNVCHLRIGK) was confirmed by chemical synthesis. The synthetic product exhibited the same physiological activity and identical molecular mass (3,933 Da) as the native toxin. Except the location of six cysteine-residues the toxin has no sequence homology to other known peptides from *Conus* venoms and hence represents a new family of potassium channel blocking conotoxins.

AN EXPERIMENTALLY-BASED MODEL OF THE 3D STRUCTURE OF A TOXIN-POTASSIUM CHANNEL COMPLEX.

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BgK isolated from *Bunodosoma granulifera* is a toxin that blocks the homotetrameric Kv1.1, Kv1.2 and Kv1.3 channel subtypes with high though different affinities. The sites by which this toxin blocks these channels have been identified, involving respectively 5, 7 and 11 residues. Among these, Lys-25 and Tyr-26 constitute a strong binding dyad to all channels and though Ser-23 is also involved in binding to the three channels it plays a more important role with Kv1.3 than with the other two channels. To understand, at a molecular level, how BgK blocks these channels, we have undertaken a series of mutations in the homotetrameric Kv1.1 channel and performed double cycle mutations with five BgK mutants, using patch-clamp experiments. The channel mutants are Y379H, S369T, S357N, E353S, in which the introduced mutations correspond to the residues found in Kv1.3, and D361N. Toxin mutations include K25A, Y26A, which affect binding to the three channels, F6A, H13A which only affect binding to Kv1.2 and Kv1.3, and N19A which only affects binding to Kv1.1 and Kv1.3. Based on the coupling energies determined between toxin and channel residues, and using a model of Kv1.1 elaborated from the X-ray structure of the bacterial KcsA channel (Doyle et al., Science, 280, 69-77, 1998), we have built 12 models of lowest energy that depict the structure of the Kv1.1-BgK complex. The data show that the key lysine 25 plugs into the pore of the channel. They also explain how the conserved and variable toxin residues interact with the channel, and offer an explanation as to how BgK can bind to three channel subtypes and how structurally different toxins can block the same channel.

THE CALCIUM CHANNEL-BLOCKING CONOPEPTIDE, ZICONOTIDE: A POTENT NEW ANALGESIC DRUG.

George P. Miljanich.

Elan Pharmaceuticals.

Ziconotide, a 25-aminoacid omega-conopeptide, is the first in a new class of analgesics. The peptide (a.k.a. SNX-111 and omega-MVIIA) was originally isolated from the venom of the marine snail, *Conus magus* and inhibits neurotransmitter release at a subset of synapses by selective blockade of presynaptic N-type calcium channels. The distribution of N channels in the nervous system defines the analgesic efficacy, safety profile, and route of administration of ziconotide. For example, N channels are localized to spinal synaptic layers receiving input from pain-sensing neurons. Thus, intraspinally administered ziconotide potently suppresses pain behavior in rat models of pain. At doses significantly above those that are minimally analgesic, intraspinal ziconotide evokes supraspinally-mediated tremor in rats, presumably due to inhibition of brain N channels. In these pain models, the drug is more potent than morphine and does not elicit tolerance. Pre-clinical studies of ziconotide were largely predictive of ziconotide's effects on humans. Ziconotide has completed two pivotal Phase III clinical studies involving several hundred patients suffering from severe pain associated with cancer, AIDS, and other syndromes. These patients had failed to obtain adequate pain relief from any other therapy. Despite drawing from among the most difficult to treat population of pain sufferers, a substantial fraction experienced significant pain relief from intraspinal ziconotide. Some patients reported supra-spinally mediated side effects. No tolerance has been observed, even in patients treated for more than three years. Ziconotide awaits marketing approval from the Food and Drug Administration.

JARARHAGIN: STRUCTURAL ASPECTS AND INVOLVEMENT IN LOCAL EFFECTS OF *Bothrops jararaca* SNAKE VENOM.

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Jararhagin is a hemorrhagic metalloproteinase/disintegrin isolated from *Bothrops jararaca* venom. This toxin presents structural similarity with mammalian matrix-degrading metalloproteinases (MMPs) and a family of proteins present on the surface of mammalian cells, known as ADAM (A Disintegrin And Metalloproteinase) protein family. Jararhagin MMP-like activity has been correlated to the hemorrhagic property of the toxin and its disintegrin activity to the selective inhibition of $\alpha 2\beta 1$ integrin binding to collagen thus inhibiting collagen-induced platelet aggregation. Moreover, jararhagin presents structural and functional similarity with TACE (ADAM 17 - TNF-α convertase), and may interfere with neutrophil migration in extra-vascular tissues that also depends on binding of $\alpha 2\beta 1$ integrin to collagen. Our recent studies have been focused on the effect of jararhagin in the local inflammatory reaction induced by *B. jararaca* venom and the correlation of our findings to structural motifs of the toxin. Injection of jararhagin in mouse subcutaneal tissues induces a dose and time-dependent leukocyte accumulation, which occurred only in the presence of resident macrophages. This effect was correlated with an enhancement of the number of roller but not adhered leukocytes in post-capillary venules and the local production of pro-inflammatory mediators as IL-6, IL-1β and TNF-α, detected by ELISA and RT-PCR on culture of peritoneal adherent cells and in the site of jararhagin injection into experimental animals. Interestingly, these effects are apparently independent on jararhagin catalytic activity and imply in binding to inflammatory cells thus suggesting the participation of jararhagin disintegrin domain. This hypothesis has been approached by testing biological activities of disintegrin and cysteine rich domains of jararhagin obtained in the native and recombinant forms, and neutralizing ability of polyclonal or monoclonal antibodies specific to each domain of the molecule.

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TOXIN STRUCTURE AND FUNCTION IN THE GENOMICS ERA.

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Genome sequences have been completed for several animals and plants, as well as numerous microorganisms. It is only a matter of time before genome sequences become available for venomous species, and proteome analyses are already being undertaken on various venom extracts. As we attempt to define the function of polypeptides and proteins identified in such studies, structural studies have an important role to play.

These developments raise the questions 'How much does the structure of a polypeptide or protein toxin contribute to an understanding of its toxicity?' and 'What impact will the rapidly developing field of structural genomics have in this area?'. These questions will be addressed by considering two examples from our own work. The first concerns a group of polypeptides that adopt the 'inhibitor cystine knot' structure [1]. This structural motif occurs in globular polypeptides and proteins from a range of phylogenetically diverse organisms, including fungi, plants, cone-shells, scorpions and spiders; it is essentially a small, well-defined scaffold that has been used by Nature for a variety of functions, but most commonly the inhibition of ion channels and enzymes. It follows that identifying this structure in a new polypeptide does not guarantee that the function of this polypeptide can be inferred beyond the broad generalisation that it may block an ion channel or enzyme of some description. This highlights the absolute requirement for functional studies in characterising any new protein, toxin or otherwise.

In the second example, the problem of proteins that act on, or are part of, biological membranes will be discussed. Some 30% of the proteins encoded by most genomes are anticipated to be membrane proteins. Moreover, among polypeptide and protein toxins, membrane-active toxins that act by disrupting cell membranes represent a major category, ranging from polypeptides such as melittin and the magainins to large proteins such as perfringolysin O, which forms massive oligomeric pores in cholesterol-containing membranes. The challenges of defining the relationship between structure and function in membrane proteins will be illustrated for equinatoxin II. This protein [2] is a potent cytolysin, and the structure in solution [3,4] provides valuable clues as to how it lyses cell membranes, while still leaving many questions unanswered.

Thus, in the two examples described here, the answer to the questions posed above are that the structures are necessary but not sufficient for understanding each toxin's mechanism of action. Structural genomics analyses of toxic proteins will leave significant gaps in our knowledge of how they work, at least in the immediate future.

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SEVERE BOTHROPIC ENVENOMATION. CURRENT CLINICAL, EPIDEMIOLOGICAL AND THERAPEUTIC ASPECTS IN COLOMBIA.

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In Colombia, 2000-3000 snakebites are yearly reported, 500-700 in Antioquia and Chocó, for a fitted incidence of 21 and 38 cases/100,000 rural inhabitants, respectively. Most of the bites (90-95%) are inflicted by *Bothrops* and *Porthidium* spp., specially by *B. atrox asper* (50-70%) and *P. nasutum* (15-30%) in northwestern Colombia. The highest incidence occurs in male 15-44 years old (53.7%), bitten in the lower extremities (71%); 28-33% are in children, 14% at their dwellings. From 50% to 60% of the patients seek medical attention after 6 hr of the bite. Thus, they are initially attended by traditional healers and arrive at the hospitals with moderate (35-40%) or severe (15-38%) bothropic envenomation, 12% of the cases being then remitted to other health institutions by lacking of antivenoms or by complications (16%). Geographical difficulties, the insufficient production and supply of antivenoms and the lack of official epidemiologic surveillance, are also some of the problems associated with the high mortality rate (5%) and of sequelae (6%) for *Bothrops* bites in the country. The clinical features of those envenomations include edema (95%), local hemorrhage (34%), blistering (12%), necrosis (10%), defibrination (62%), thrombocytopenia (31%), gingival bleeding (23%), hematuria (25%), hypotension (14%) and other hemorrhage distant from the bite site. Acute renal failure (11%), cellulitis/abscess (11-18%), compartment syndrome (3%), hemorrhage in the central nervous system (2-3%) and abortion or *abruptio placentae* may arise as complications. Severe envenomation is characterized by non-clottable blood and local necrosis or local swelling extending beyond the bitten limb, local and systemic bleeding, and hypotension or renal failure or central nervous system hemorrhage. After three randomized clinical trials performed in the region, including serum venom and antivenom measurements, and using antivenoms from Brazil, Costa Rica and Colombia, antivenom doses currently recommended are 2, 4 and 6-9 vials for mild, moderate or severe bothropic envenomations, respectively, the highest dose also when the snake is > 100 cm body length. Ancillary treatment must include the administration of plasma expanders to correct the hypovolemia, broad spectrum antibiotics (e.g., Sulbactam/Ampicillin; Clindamycin + third generation Cephalosporins or Fluoroquinolones; TMP-SMZ) for all moderate or severe cases, the tetanus prophylaxis (second day) and the hourly urine output measurement. Additionally, the blood platelets and coagulation status monitoring at 12, 24, 48 and 72 hr. One sufficient antivenom dose will totally restore blood coagulation status no later than 12-24 hr after the onset of serotherapy. Bleeding different of hematuria, must stop within the first 6-12 hr of treatment. By monitoring the serum creatinine and electrolyte concentrations, as well as the acid-base status, all of this may contribute to indicate the dialysis in patients with renal failure, in some of which plasma expanders, furosemide (1-2 mg/Kg) and dopamine infusion (2.5-5.0 µg/Kg/min) can fail. If a compartment syndrome is suspected, and the intracompartmental pressure is above 30 mm/Hg (children) or 45 mm/Hg (adults), the i.v. administration of Mannitol (1-2 g/Kg over 30-60 min) can do unnecessary the fasciotomy. Blisters and necrotic skin debridement and amputations, are performed 3-5 days after the bite; skin grafts and rehabilitation after the second week of treatment.

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THE NATURAL COMBINATORIAL CHEMISTRY STRATEGY OF THE WEB-SPIDERS:A CHEMODIVERSITY OF ACYLPOLYAMINETOXINS.

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The venoms of *Nephilinae* web-spiders contain a series of acypolyaminetoxins in their venoms, as a very complex mixture of similar compounds occurring each one at picomol level. These compounds constitute a well know class of neuroblockers, acting on NMDA-type glutamate receptors.

By using a combination of NMR, MALDI-TOF/MS and HPLC-continuous flow (FRIT) FAB-MS and MS/MS under high energy CID conditions in a four sector tandem mass spectrometer, it was possible to elucidate about 62 new different acylpolyaminetoxin structures in the venoms from *Nephila* and *Nephilengys* spiders.

Thus, there are about 85 known different structures of these toxins among the *Nephilinae* spiders, whose are constituted of four parts: i) a mandatory aromatic moiety constituted by either 2,4-dihydroxyphenyl, or 4-hydroxyindole or indole; ii) a linker amino acid: asparagine; iii) a polyamine backbone, constituted by a combination of polyamines and/or amino acids: cadaverine, putrescine, diamino propane, putreanine, glycine and alanine; iv) an optional tail, which generally is constituted by a basic amino acid: either arginine, or lysine or ornithine.

Such wide chemical diversity of acylpolyaminetoxins may be related to the fact that the web-spiders are polyafagous animals, which change their venom composition seasonally to paralyse the most abundant prey under each different enviromental conditions. Thus, the *Nephilinae* spiders are able to perform a natural combinatorial chemistry for the biosynthesis of acylpolyaminetoxins in order to attend the demand for a large number of toxin structures according to their very complex preying behavior.

NATURAL PROTEIC INHIBITORS OF PHOSPHOLIPASE A_2 AND MYOTOXINS: INSIGHTS INTO STRUCTURAL AND FUNCTION RELATIONSHIPS.

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Several PLA_2 inhibitors (PLIs) have been isolated from blood of venomous and non-venomous snakes. They have been associated with the resistance of the snake to its own venom or to the deleterious effects of their venom PLA_2 components, mainly neurotoxic and myotoxic activities. All PLIs isolated so far are oligomeric acidic glycoproteins, classified in three groups based on the homology of their amino acid sequence: PLIα, homologous to the carbohydrate recognition domain (CRD) of C-type lectins (also present in M-type PLA_2 receptors); PLIβ, which has 33% identity to the serum human protein leucine-rich α_2-glycoprotein; and PLIγ characterized by the presence of two tandem patterns of cysteine residues constituting two internal typical three-finger motif. Independently of their molecular structure, all PLIs studied to date have shown complex formation with the PLA_2 that they inhibit. In the particular case of the Crotoxin Inhibitor from *Crotalus* Serum (CICS), it was shown that it formed a soluble inactive complex with the catalytic subunit (CB or CbII) of heterodimeric neurotoxins, such as crotoxin, mojave toxin and CbICbII, but not with non-catalytic subunits, such as CA or CbI. CICS displaced the non-enzymaticaly active subunit from the complex, inhibited the PLA_2 catalytic activity of these toxins and neutralized their neurotoxic and lethal activities. PLIα, PLIβ and CICS are present in *Viperidae* snakes as well as the PLA_2 that they inhibit. Other PLIγ present a broader specificity because they can inhibit PLA_2 from groups I, II and III. Some specific domains or motifs of the PLIs have been involved in the binding with the PLA_2, as CRD, leucine-rich repeats and three-finger motif. It was shown that some PLIs inhibit the myotoxic effects of PLA_2 myotoxins. More recently, a novel myotoxic inhibitor (DM64) from the immunoglobulin supergene family was isolated from *D. marsupialis* serum. It inhibited the myotoxic activity of myotoxin I (D49, enzimaticaly active) and myotoxin II (K49, inactive) isolated from *B. asper* venom but did not inhibit the PLA_2 activity when present, showing that myotoxicity and catalytic activity are independent phenomena.

NOVEL CLASSES OF TOXIC PEPTIDES IN THE VENOM OF SCORPIONS: STRUCTURE AND FUNCTION.

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Scorpion venoms contain an immense number of bio-active peptides, estimated to be in the range of 100,000 (Eur.J.Biochem. 264, 287-300, 1999). The best known are the α-and β-toxins that modify Na^+-permeability of excitable cells, the K^+-channel blockers and the Cl^- and Ca^{2+}-channel specific toxins. A common structural feature of these toxic peptides is a segment of α-helix and 2-3 segments of antiparalel β-sheets, stabilized by two constant disulfide bridges. However, in recent years a number of new classes of peptides with different activities have been reported such as: maurocalcine, kurtoxin, phospholipin, hadrurin, scorpin, ergtoxin, BmK AEP and Tc1. Our group found CII9 and Cn11, two Na^+-channel toxin-like peptides with a distinct mode of action (unpublished). Many other peptides are present in scorpion venoms, for which we do not know neither their structures, nor their functions. This will be discussed during the presentation.

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A RETROSPECTIVE OF TOXINOLOGICAL RESEARCH IN THE DEPARTMENT OF PHARMACOLOGY AT UNICAMP, CAMPINAS, BRAZIL.

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The Department of Pharmacology at the State University of Campinas (UNICAMP) was founded by Dr. Oswaldo Vital Brazil in 1964. Initial work under Dr.Vital Brazil in the 1960s and 1970s involved pharmacological studies of crotoxin and crotamine from the venom of *Crotalus durissus terrificus*, and the discovery of convulxin. Work on coral snake (*Micrurus*) venoms in the 1970s and 1980s established the mechanism of action for several species. In the early 1980s, research on the venom of *Bothrops jararacussu* resulted in the first pharmacological characterization of a bothropic myotoxin (bothropstoxin-I). These studies have been extended to other *Bothrops* species, including the Caribbean species *B. lanceolatus*. Recent studies of the Duvernoys gland secretion of the back-fanged colubrid *Philodryas olfersii* have lead to the isolation and characterization of a myotoxin.
Invertebrate venoms have also been studied, starting with scorpion (*Tityus serrulatus*) venom, but later involving spider (*Phoneutria nigriventer*, *Theraphosa* sp.), caterpillar (*Lonomia obliqua*) and wasp venoms. Components isolated from these venoms include a novel tissue-kallikrein activator and various vasoactive peptides from *P. nigriventer* venom and, more recently, a new cardiotoxin(s) from *T. serrulatus* venom. Notable contributions to clinical toxinology by Dr. Vital Brazil include the use of neostigmine to antagonize the neurotoxic effects of certain coral snake (e.g. *M. frontalis*) venoms and, more recently, the use of $MgCl_2$ to treat neurotoxicity after envenomation by scorpions. Numerically, what started as one research group under Dr. Vital Brazil has now expanded to five groups with numerous post-graduate students working in different areas of toxinology.

Financial support: FAEP

IDENTIFICATION OF *Enterobacter* BACTERIA AS SAXITOXIN PRODUCERS IN CATTLE'S RUMEN AND SURFACE WATER FROM VENEZUELAN PLAINS.

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We have previously shown that a paralytic toxin able to block sodium channels in nerve is associated with a cattle's disease known as bovine paraplegic syndrome (BPS) (Sevcik *et al.*, 1993). This toxin has recently (Sevcik *et al.*, 1998) been identified as saxitoxin (STX) using HPLC both labeling the compound with picrylsulfonic acid on μBondapack™ Phenyl columns, or using the method of Oshima *et al.*, (1987) for detecting saxitoxin (STX) and related paralytic shellfish toxins. In recent experiments we were also able to collect and cultivate facultative anaerobic bacteria growing on rumen, grass and ponds of corrals with high incidence of BPS; the cultured bacteria produce compounds indistinguishable from STX under both HPLC procedures described above. Two strains of bacteria from field samples have been identified using standard biochemical criteria (API 20-E kit, bioMérieux Inc., Montanlieu, France) and using gas chromatography of bacterial lipids (Microbial ID Inc., Newark DE). The GC method produces a *microbial similarity index* (msi) which relates the lipid profile of the unknowns with known bacteria. Both techniques identified two species from the *Enterobacter* genus, *E. asburiae* (msi 0.779 in water samples, 0.736 in ruminal liquor) and *E. clocae* (msi 0.764 in ruminal liquor, 0.732 in water samples) as the toxin producers. The closeness of the msi is such that the two strains may actually be any of the two species mentioned above or another unknown species of the genus *Enterobacter* very closely related to them; yet the last option seems unlikely.

PROTEIN ENGINEERING AND THE EVOLUTION OF A VACCINE.

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Throughout the development of a biopharmaceutical, emphasis must be placed on the quality attributes of the product. Quality, safety and effectiveness must be designed and built into the product. Quality cannot be tested or inspected into a finished product. Each stage of the manufacturing process must be controlled, so as to maximize the probability that the finished product will meet all quality and design specifications.

Title 21 of U.S. CODE OF FEDERAL REGULATIONS ("21 CFR") states the purpose of Good Manufacturing Practices (GMPs) is "to assure that all pharmaceutical, biologic, diagnostic, and medical device products meet all of the requirements of the Federal Food, Drug, and Cosmetic Act as to safety, and efficacy and have the identity and strength to meet the quality and purity characteristics which they purport to have".

From discovery and concept exploration through program definition, risk reduction, engineering, manufacturing, and production.

This presentation will focus on the utility of protein engineering

The expectation from FDA is that a recombinant vaccine shall be well-characterized in terms of its purity, identity, efficacy, potency, consistency, stability, and most importantly, its safety.

Clostridium perfringens IOTA TOXIN: THE UNVEILING OF A BINARY BEAUTY.

B. G. Stiles, J.-C. Marvaud, M. L. Hale, T. Smith, L. A. Smith and M. R. Popoff.

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Clostridium perfringens is an anaerobic, Gram positive bacterium commonly associated with gas gangrene, human food poisoning, and animal enterotoxemias. One of the major lethal and dermonecrotic toxins produced by this ubiquitous microorganism is iota, which shares amino acid homologies and epitopes with other binary, nonlinked proteins produced by spore-forming bacilli like *Bacillus anthracis* (anthrax toxin), *Clostridium botulinum* (C2 enterotoxin), and *Clostridium spiroforme* (iota-like toxin). Iota toxin consists of Ib, which binds to a cell-surface protein, and a transferase (Ia) that docks to cell-associated Ib, enters a target cell, and mono-ADP-ribosylates intracellular actin thus disrupting the cytoskeleton. Ib is produced as a protoxin activated by serine-type proteases, which liberate a 20 kD N-terminal peptide and enables Ib to oligomerize and then dock with Ia. Truncated variants of activated Ib were tested for binding to Vero cells and docking with Ia via fluorescence-activated cytometry. Deletion of just ten C-terminal residues (656-665) from Ib inhibited binding to the cell surface and Ib peptides (> 200 C-terminal residues) prevented iota cytotoxicity. The N-terminal residues (1 - 27) of Ib were important for Ia docking but peptides from this region did not prevent iota cytotoxicity. Mapping of Ib with monoclonal antibodies revealed two distinct neutralizing epitopes within the C-terminus. One antibody prevented Ib binding to the cell surface. The other did not inhibit Ib binding but prevented cell-surface oligomerization. The N-terminal binding antibodies had no effect on iota cytotoxicity or oligomerization. Overall, these studies provide useful clues for understanding bacterial binary toxins, especially iota toxin and its potential as a biological tool for delivering therapeutics

STRUCTURAL REQUIREMENTS OF *ENDO*POLYGALACTURONASE FOR THE INTERACTION WITH PGIP (POLYGALACTURONASE INHIBITING PROTEIN).

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To invade a plant tissue, phytopathogenic fungi produce several cell wall degrading enzymes; among them, *endo*polygalacturonase (PG) catalyses the fragmentation and solubilisation of homogalacturonan. Polygalacturonase-inhibiting proteins (PGIPs), found in the cell wall of many plants, counteract fungal PGs by forming specific complexes with them. We report here the crystal structure, at 1.73 Å resolution, of PG from the phytopathogenic fungus *Fusarium moniliforme* (*Fm*PG). Several amino acids of *Fm*PG were mutated and their contribution to the formation of the complex with PGIP-2 from *Phaseolus vulgaris* was investigated by surface plasmon resonance. The residues Lys269 and Arg267, located inside the active site cleft, and His188, at the edge of the active site cleft, are critical for the formation of the complex which is consistent with the observed competitive inhibition of the enzyme played by PGIP-2. The replacement of His188 with a proline or the insertion of a tryptophan at position 270, variations that both occur in plant derived PGs, interfere with the formation of the complex. We suggest that these variations are important structural requirements of plant PGs to prevent PGIP binding.

COMPLEX INTEGRATED PATHOPHYSIOLOGY OF *Carybdea alata* TOXINS.

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Hawaiian box jellyfish (*Carybdea alata)* venom contains multiple and potentially synergistic biologically active compounds ranging from peptides and proteins to bioactive lipids and complex carbohydrates. Since envenomation involves not only the delivery of venom into the targeted prey, but also physical perforation of prey tissue by nematocysts tubules, we have conducted biochemical purification and characterization of both the venom as well as tubule-associated structural-compounds to assay for toxicity. We first isolated and characterized a novel 42 kDa hemolytic protein (CAH1) from the venom of the Hawaiian box jellyfish (*Carybdea alata)*. The activity exhibited lectin-like properties in that hemolysis was inhibited by D-lactulose and certain other sugars. We have also isolated toxic bioactive lipid compounds. Recent findings indicate that other venoms also contain similar compounds and suggest their role in the pathophysiology associated with envenomation. Additionally, we have begun to characterize the composition of the tubules and find evidence for potential antigenicity. We suggest that this type of integrated analysis of toxicological responses to envenomation may provide new insights into the clinical management of cnidarian stings, as well as the biological selection of prey species.

POSTER

ABSTRACTS

P1

DIFFERENTIAL EFFECTS OF POSITIVE AND NEGATIVE STIMULI ON THE CARDIAC ACTIVITY OF THE CRAB *Hepatus pudibundus*.

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The crab *H. pudibundus* has a variable heart activity, with episodes of cardiac arrest interspersed with periods of constant beating. As in other decapod crustaceans, the heart frequency (HF) can be modified by the presentation of chemical stimuli to the intact animal. We have studied the effect of positive (clam or fish extracts) and negative (crustacean digestive secretion) stimuli on the cardiac activity of *H. pudibundus*. Our results indicate that positive stimuli accelerate the beating heart and make a quiescent one start beating. By contrast, negative stimuli stop a beating heart and have no effect on a quiescent one. The control stimulus (filtered sea water) is perceived as negative, because it stops the heart. This last result contrasts with those obtained from two other braquiuran species (*Callinectes danae* and *Ocypode quadrata*). When mixtures of both types of stimuli are applied the final result depends on their ratio, changing from a negative- to a positive-stimulus one as the mussel extract proportion increases. However, the presence of the digestive secretion seems to be perceived even when diluted 2500 times in mussel extract, because the tachycardia induced is milder and less-lasting than that induced by the mussel extract alone. Our results indicate that the cardiac activity of *H. pudibundus* may be used to monitor how an unknown substance is perceived by organisms in the marine environment.

P2

INFLUENCE OF BUFFER COMPOSITION ON CHROMATOGRAPHIC BEHAVIOR OF *Bothrops erythromelas* VENOM CONSTITUENTS ON SUPERDEX 75.

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Bothrops erythromelas, a small terrestrial pitviper, is endemic to northeastern Brasil. Despite its diminutive size, it is responsible for a disproportionate number of human envenomations in this region, resulting in profound hemodynamic disturbances. In a chromatographic method modification intended to preserve protease activity, 2 mM $CaCl_2$ was added to the gel filtration buffer [50 mM Tris/HCl/150 mM NaCl (pH 8.0)], in lieu of an equimolar portion of NaCl. This minor compositional change induced profound differences in the elution profile of *B. erythromelas* venom from Superdex 200. For this reason the influence of buffer composition on chromatographic behavior was investigated using a Superdex 75 HR 10/30 column. Phospholipase (PLA) was used as a marker because *Naja atra* PLA had previously been observed to interact hydrophobically with this resin. PLA elution volumes increased with decreasing buffer pH. Sodium acetate buffers reduced PLA hydrophobicity relative to sodium acetate/NaCl buffers, apparently due to ion pairing capacity of acetate ion. Addition of 20% acetonitrile to the Tris buffer with $CaCl_2$, reduced the hydrophobic interaction of *Bothrops erythromelas* PLA so significantly that PLA elution was non-overlapping in the two buffers. Other venom constituents, including hemorrhagic metalloproteases were similarly affected.

P3

EFFECT OF SURAMIN ON MYOTOXICITY OF DIFFERENT CROTALID SNAKE VENOMS FROM NORTH AND SOUTH AMERICA IN MICE.

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We investigated the protection by suramin of the myotoxic effect of seven different crotalid snake venoms from North and South America (*A. c. laticinctus*; *C. v. viridis*; *C. d. terrificus*; *B. jararacussu*; *B. moojeni*; *B. alternatus*; *L. muta*) in mice. The myotoxicity was evaluated in vivo by i.m. injection of venom (0.5 -1.0 mg/kg) dissolved in physiological saline solution (PSS, 0.1 ml) either alone, or after 15 min. of pre-incubation with suramin or 15 minutes before the i.v. injection of suramin (1mg/kg). Before and 2 h after the i.m. injection the animals were lightly anesthetized with diethyl-ether and the blood was collected by orbital puncture. The plasma was separated by centrifugation and stored at 4°C for subsequent determination of creatine kinase (CK) activity. The increase of plasma CK was significantly reduced (37-76 %) by pre-incubation with suramin, except for *B. alternatus* and *L. muta* venoms. Post-treatment did not protect against *C. v. viridis*, *L. muta*, *B. moojeni* or *B. alternatus* venoms and partially protected (34-51%) against the myotoxicity of *B. jararacussu*, *A. c. laticinctus* and *C. d. terrificus* venom. These results showed that suramin has antimyotoxic effect against some American crotalid snake venoms.
Financial support: FAPERJ; PRONEX (No. 1.96.0888.00), FUJB-UFRJ and CNPq.

P4

THE ANTI-SNAKE VENOM ACTIVITIES OF THE PLANT EXTRACT Pb: *IN VITRO* AND *IN VIVO* STUDIES.

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Pb is a methanol extract of a medicinal plant used in the South East of Nigeria for the treatment of snakebite. It significantly ($p<0.001$) protected the chick biventer cervicis (cbc) muscle from *N. nigricollis*-induced inhibition of externally evoked twitches. The effect was observed when extract was added to the bath 3 to 5 min before or after venom. Pb also increased postsynaptic sensitivity to Ach, carbachol and KCl that is normally blocked by *N. nigricollis* venom. It significantly protected C2C12 murine cells against the cytotoxic effects of *N. nigricollis* and *E. ocellatus* venoms. Chicken embryos exposed to lethal concentrations of *E.ocellatus* venom and varying concentrations (5 and 10 ug/1.5 ul) of Pb remained alive for over 24 h. It did not protect mice injected i.p. with 2.5 and 5.0 mg/kg of *N. nigricollis* and *E. ocellatus* respectively but gave 40% protection after extract and venom were pre-incubated for 30 min before injecting the mixture.

P5

MAPK IS INVOLVED IN PARDAXIN-INDUCED DOPAMINE RELEASE FROM PC12 CELLS.

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Pardaxins, an ionophore-peptide neurotoxin isolated from the fish *P. marmoratus*, induces neurotransmitter release from neuronal preparations by both calcium-dependent and calcium-independent mechanisms.The aim of the present study was to investigate the role of MAPK in Pardaxin-induced dopamine release in PC12 cell lines. Time-course experiments indicated that Pardaxin stimulated MAPK1 and MAPK2 whithin 5-15 min, measured with a dual phospho-MAPK antibody. Pardaxin stimulation of MAPK activity was calcium-dependent and followed by MAPK translocation to the nucleus. This effect was temporally related to Pardaxin-induced exocytosis and was blocked by PD-98059, a selective MEK inhibitor. These results suggest an essential role of MAPKs in Pardaxin-induced neurotransmitter release, most probably by involving phosphorylation of proteins regulating exocytosis.

P6

THE COMPLEX EFFECT OF *Tityus discrepans* SCORPION VENOM ON HEMOSTATIC MECHANISM INVOLVES: DEGRADATION OF FIBRINOGEN α CHAIN, THE PRESENCE OF PLASMIN-LIKE FRACTIONS AND/OR PLASMINOGEN ACTIVATORS, AND INHIBITORS OF PLASMIN AND/OR PLASMINOGEN ACTIVATORS.

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We studied in vitro the activity of *Tityus discrepans* (*Td*) venom on the coagulation cascade. *Td* whole venom (WV) was fractionated by molecular exclusion HPLC (Protein-Pak™ 125 column, 7.8 x 300 mm, Waters) eluting at 0.5 ml/min with 20 mM $CH_3.COONH_4$ at pH 4.85. Six fractions (FI through FVI) were separated with retention times ranging from 12.8 to 31 minutes. Our results show that *Td* venom contains pro- and anti-coagulant components separable by HPLC. Partial thromboplastin time (PTT) was shortened by both WV, and by pooled isolated fractions (PIF) in concentration ranging 78 to 625 μg/ml, but was prolonged at concentrations between 700 and 1000 μg/ml. Fraction I (FI) shortened PTT at concentrations between 10 and 20 μg/ml but fraction VI (FVI) prolonged PTT when 16 to 650 μg/ml were used. It does seem thus, that FI produces shorting in PTT while FVI produces the prolongation observed with WV. Plasma recalcification time (PRT) was prolonged by WV and PIF in a dose dependent manner, this effects seems to be due to fractions II, III and VI. PRT was shortened in a dose dependent manner by FI and FV, but this effect was masked in WV and PIF by the other fractions. Only FIV had a dual effect since it was able to prolong PRT at low concentrations and to reduced it at high concentrations. Prothrombin time (PT) was prolonged by WV and fraction II at concentrations from 33 to 333 μg/ml. The α chain of fibrinogen (6 μg/μl) was degraded by 0.1μg/μl of WV after incubating during 5 min, at 37°C. The fibrinolytic activity measured by fibrin plate in presence of plasminogen suggest that WV contain a plasmin-like or/and plasminogen activator with an inhibitor plasmin activity.

PRODUCTION OF JAR49, ERISTOSTATIN AND THE CHIMERICAL ECD-ERISTOSTATIN IN FUSION WITH *Escherichia coli* ALKALINE PHOSPHATASE (AP).

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Eristostatin, a 49-residue RGD-disintegrin, binds to αIIbβ3 integrin, thus inhibiting ADP-induced platelet aggregation. Jararhagin, a multidomain metaloprotease, shares 74% of sequence similarity with eristostatin within the disintegrin domain. However, instead of the RGD motif, this domain contains an ECD sequence thought to be responsible for binding to the α2β1 integrin.
Our goal is to develop molecular tools selective for different integrins to be used for integrin mapping during tumor development. For this reason we have cloned eristostatin and its jararhagin analogous sequence (jar49), in the pLIP6 vector, which allows the expression of foreign proteins in fusion with AP. After induction eris/AP and jar49/AP showed AP activities of 4 and 28 U/mL, respectively. The platelet aggregation test showed 100% of inhibition by eris/AP and a partial inhibition by jar49/AP. We used the bifunctional activity of our proteins in a dot blot test, which showed the binding of eris/AP to platelets. These data support our hypothesis of using hybrid disintegrin/AP molecules as biological tools. Furthermore we have substituted the eristostatin RGD loop by their jar49 counterpart, in order to establish the disintegrin potency of the ECD sequence. The ECD-eris/AP fusion protein showed AP activity, but failed in binding to platelets in the dot blot test. The different reasons susceptible to explain that result and the perspectives we plan to give to that project should be discussed.
Financial support: FAPESP.

P8

ULTRASTRUCTURAL ANALYSIS OF THE EFFECT OF HEPARIN ON MICE MUSCLE REGENERATION AFTER BEING DAMAGED BY *Bothrops* VENOM .

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Objectives: The aim of this study is to analyze the ultrastructural aspects of the effect of treatment with heparin on the regeneration of damaged mice's skeletal muscle after the injection of *Bothrops jararacussu* venom.
Methods and Results: Adult mice weighing 20-25 g were anesthetized with ether and injected with crude venom (1 mg/kg) over the EDL (Extensor digitorum longus) muscle of the right posterior limb. The mice were separated in groups and each group received treatment, by intravenous route with either heparin (H - 10 mg/kg), or low molecular weight heparin (LMWH - 10 mg/kg), or specific antivenom (AV - 0,1 μL/g) at 15 min. and 4 h after the injection of the venom. 21 days after the injection of the venom the animals were killed, under anesthesia with ether, and the EDL muscles were dissected out and fragments were immersed in fixative solution for 2-4 h. Then the fragments were washed in buffer and posfixed for 1 h in 1% OsO4. The tissue was dehydrated in acetone and embedded in Polybed 812 resin. Ultrathin sections (70-90 nm) were obtained and observed on electron microscope. Our results show that while this treatments promoted a good muscle regeneration, with the organization of regenerated fibers being similar to the control, the animals that not receive any treatment exhibited a complete desorganization of muscle cells. Conclusions: These data indicate that heparin improve the regeneration of EDL muscle damaged by *B. jararacussu* venom.
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SCORPION ENVENOMATION IN MÉRIDA, VENEZUELA: CARDIOVASCULAR MANIFESTATIONS AND POSSIBLE MECHANISMS.

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Eighteen children were referred to the Pediatric Emergency of the University of Los Andes Hospital between january 1999 and august 2000, with the clinical diagnosis of scorpion envenomation. Sixteen of them came from the southwest section of the state of Mérida. The clinical manifestations of the scorpion envenomation and the electrocardiographic and echocardiographic abnormalities were assessed. Plasma norepinephrine levels were measured by high-pressure liquid chromatography, on admission and 24 hours after hospitalization. Ten of these patients had variable degrees of pulmonary edema and high plasma norepinephrine levels (1263 ± 817 pg/ml, M ± SD). Seven of them had left ventricular wall motion abnormalities and moderate to severe left ventricular systolic dysfunction. A significant and direct correlation ($r = 0.76$, $p<0.01$) was found between the time interval for antivenin administration and the absolute variation in plasma norepinephrine levels, at 24 hours after admission. The presence of left ventricular wall motion abnormalities and the very high levels of plasma norepinephrine strongly suggest that in these patients pulmonary edema is cardiogenic and secondary to the toxic effect of catecholamines on the ventricular myocardium. Furthermore, these results indirectly suggest that, the time window for antivenin administration, is a critical factor in the medical management of this environmental emergency.

P10

SHORT NEUROTOXINS FROM THE VENOM OF *Micrurus pyrrhocryptus*.

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Micrurus pyrrhocryptus is one of the venomous snakes of sanitary risk in Argentina and the Elapid of widest distribution. Despite human accidents involving *Micrurus* being lower than those from the other genera, they are of high life-risk, in all cases. The *M. pyrrhocryptus* venom has a potent LD50 (1.3 μg / g of mouse) and its injection causes a clinical picture of severe neurotoxicity. On account of this, we have begun to characterize the venom of Argentine *M. pyrrhocryptus* specimens. Material was ultrafiltered by using Centricon YM-10 filter units (cut off: 10000). The venom as well as its lower and higher MW fractions were immediately frozen and stored at -70°C. Fractions were then analyzed by SDS-PAGE stained with Coomassie Blue or silver staining and molecular mass of the individual components was determined in order to study, first, the presence of short neurotoxins. Since bands of MW ranging between 6 and 8 kDa were detected, proteins were electroblotted onto a PVDF membrane but the material amount was not enough for unambiguous sequence determination. Samples were then concentrated in a Speed Vac and RP-HPLC was performed. A C18 Vydac column was used and the elution was initially isocratic and then a gradient of ACN in 0.1% TFA was utilized. Each peak was submitted to SDS-PAGE. The N-terminal sequence (28 residues) allowed us to show the presence of a short neurotoxin in the protein mixture and homology studies indicated an important sequence similarity with that of the DE1 polypeptide from King Cobra venom.

P11

CROSS REACTIVITY OF THE FABOTERAPIC CORALMYN® TOWARD DIFFERENT SNAKES POISONS OF *Micrurus* spp.

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The *Micrurus* bites can cause death by muscle paralysis and further respiratory arrest a few hours after envenoming. We works with different poisons of *Micrurus*, (*Micrurus nigrocinctus*, *Micrurus fulvius*, *Micrurus surinamensis* of Colombia lot 1 and *Micrurus surinamensis* of Colombia lot 2) to these venoms were carried one electrophoretic slide in SDS PAGE under native and reducing conditions. The fractionated proteins were electro-transferred to a nitrocellulose sheet by transfer BioRad sistem. On the other hand also we were carried out ELISA'S technique to determine the quantitatively recognition of the Coralmyn®, toward the different poisons in study, and we used the Prisma softwere. We could observe, the Coralmyn® is able to recognize to the poisons of the differents *Micrurus* studied and it is important since the faboterapic is only manufactured with poison of *Micrurus nigrocinctus*, but it presents an important crossed reactivity toward other species of the same agreement gender with the Western Blot analysis. The ELISAS analysis, where the one titles opposing average for the Coralmyn® toward *Micrurus nigrocinctus*, it is of 5741.5, while it stops *Micrurus fulvius* it is of 3098 and it stops *Micrururs surinamensis* it is of 981.25. The Coralmyn® can be able to use for snake envenomations with *Micrurus surinamensis* and *Micrurus fulvius* but we need to employed more dosage.

NATRIURETIC PEPTIDES FROM VENOMS.

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While natriuretic peptides were initially isolated from and thought to have a role only in circulating physiological systems, more evidence is showing that venomous animals have been creative in their use of these peptides. Both ANP-like and CNP-like peptides have been previously isolated from either the venom itself or venom gland cDNA libraries of a diverse array of animals. ANP-like include DNP from the venom of the Eastern green mamba (*Dendroaspis angusticeps*) from South Africa, and MNP identified in cDNA libraries of the Painted coral snake (*Micrurus corallinus*) from South America; CNP-like components have been found in snake venoms such as the Habu (*Trimeresurus flavoviridis*) and Indian green tree pitviper (*Trimeresurus gramineus*) from SE Asia and the Mamushi (*Gloydius blomhoffii*) also from SE Asia as well as in the venom of the Australian monotreme the Platypus (*Ornithorhynchus anatinus*). Further, CNP-like molecules have been identified in the venom gland cDNA libraries of the Jararaca (*Bothrops jararaca*) from South America. It is interesting to note that all of ANP-like molecules have been isolated from *Elapidae* snake venoms while the CNP-like molecules have been isolated from Subtle differences in primary sequences and activity *Viperidae* snake venoms. The ANP molecules have been found to be widespread and abundant in the venoms of Australian elapids. Three isoforms found in the venom of *Oxyuranus microlepidotus* were isolated and characterised. Despite the great similarity in sequence, differences in activity were evident.The nomenclature used in naming these components reflects the genus isolated from, molecule type and chronological order of isolation. As such they were named named OxyNPa, OxyNPb and OxyNPc.

P13

EFFICIENT PURIFICATION AND CHARACTERIZATION OF GRAY WOODRAT (*Neotoma micropus*) SERUM FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES.

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Gray woodrats (*Neotoma micropus*) have natural inhibitors that neutralize hemorrhagic and other proteolytic effects of snake venom. Similarly, human protease inhibitors play a key role in both physiological and pathological processes. Venom metalloproteases and natural inhibitors make excellent models for studying tumor metastasis. The present study was undertaken to isolate protease inhibitors in large quantities from *N. micropus* and determine characteristics of the protease inhibitors. A total of 38 woodrats were collected in Kleberg County, Texas, USA. Electrophoretic titration (ET) was a useful procedure for determining the PI of proteins, the surface charge ratio, the complexity of woodrat sera, and the optimal conditions for separation of serum by HPLC. Woodrat serum has different proteins that neutralize various proteolytic enzymes in *Crotalus atrox* venom. Woodrat serum did not neutralize the antifibrinolytic activity in *C. atrox* suggesting this serum does not have protease inhibitors against the fibrinolytic enzymes found in *C. atrox* venom. The sera from *N. micropus*, *Sigmodon hispidus*, and *Spermaphilus mexicanus*, cross-reacted with the monoclonal antibody suggesting they have common antigenic sites. ET profiles proved to be an excellent tool for predicting the optimal conditions for HPLC fractionation. HPLC was a powerful tool in determining proper conditions for the batch procedure. The batch method was the best way to purify proteins in large quantities. The development of this method is important because it will save time for future separations.

P14

ANTIBACTERIAL ACTIVITY OF *Bothrops* VENOMS OF ARGENTINE.

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Envenomated snakebite wounds are typically puncture wounds asociated with toxin-mediated swelling and tissue destruction. Previous studies have demonstrated potentially pathogenic aerobic and anaerobic flora of the snake oral cavity, many gram negative aerobic species. Despite these predisposing factors, human snakebite victims appear to sustain infectious complications infrequently. Although the biologic activity of *Bothrops* venoms has been extensively studied, the bactericidal activity of venoms from Argentine has not been defined previously. Some authors hypothesized that snake venom may be directly bactericidal, thus suggesting that snake venoms may decrease the risk of wound infection by direct bacterial killing. The aim of this study was investigate the antimicrobial activity of four venoms of *Bothrops* genus, from Argentine, using the Minimal Inhibitory Concentration (MIC) against standard bacterial strains and standard yeast. Concentration between 1,6 and 100 ug/ml of *Bothrops alternatus*, *Bothrops neuweidii*, *Bothrops jararaca* and *Bothrops jararacussu* venoms were tested against seven microorganisms: *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeuruginosa* (ATCC 9027), *Micrococcus luteus* (CCMA 45), *Escherichia coli* (ATCC 11105), *Listeria monocytogenes* (CCMA 454), *Bacilus subtilis* (ATCC 6633) and *Candida albicans* (CCMA Y-1). The MIC obtained for these venoms was <100 ug/ml to *S.aureus*, *P.aeuruginosa*, *M.luteus*, *E.coli*, and *L.monocytogenes* but standard strain of *B.subtilis* and *C.albicans* were resistant to all concentrations tested. *Bothrops* venoms are broadly active against aerobic gram-negative and positive bacteria; this activity may play a role in the low incidence of infection after envenomation injuries.

SCALE-UP PRODUCTION OF SALMOSIN, A NOVEL DISINTEGRIN, IN *Pichia pastoris*.

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Salmosin is a Korean snake venom derived disintegrin that antagonizes platelet aggregation (Throm. Res 91, 65, 1988), and significantly inhibits bovine capillary endothelial cell proliferation induced by basic fibroblast growth factor (bFGF) but has no effect on normal growth of cell. The bFGF induced *in vivo* angiogenesis in the choriollantoic membrane was blocked by treatment of salmosin with no effect on normal angiogenesis (Cancer Res 59, 3754, 1999). The methylotrophic yeast *Pichia pastoris* has been developed to be an excellent host for the production of foreign proteins. The most important features of the system are that proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium. The secretion signal of yeast alpha-factor has been used as a fusion partner for recombinant salmosin in expression vector pPIC9. The recombinant protein was successfully expressed as an active form by methanol induction of transformed yeast. We obtained about 1.5 g of purified recombinant salmosin from 30 liter fermentation broth. (The overall yield was about 50mg per 1liter fermentation broth). The IC_{50} values of recombinant salmosin for the inhibition of platelet aggregation and BCE cell growth were same to those of natural salmosin. Reconbinant saxatilin also has the same anti-tumor activity as natural salmosin. Here we report the efficient expression of active salmosin with no need to complicate purification steps as well as the refolding and cleavage of fusion protein.

THE CYSTEINE-RICH DOMAIN OF THE METALLOPROTEINASES ATROLYSIN A AND JARARHAGIN: ITS INTERACTION WITH PLATELETS.

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The high molecular weight metalloproteinases, Atrolysin A and Jararhagin interfere with the platelet responses to collagen. These enzymes have an N-terminus with catalytic activity, a non-RGD disintegrin-like domain and a C-terminus cysteine-rich region. We, and others, have already shown that the disintegrin-like plus the cysteine-rich region of these two enzymes, as well as the cysteine-rich region of Atrolysin A alone, play a role in the inhibition of platelet responses to collagen. It is still unclear which domain is responsible for this activity, or whether more than one domain is involved. To clarify this, we constructed synthetic peptides (cyclized, linear and some with alanine substitution of cysteine residues) based on both domains. Peptides were incubated with platelet suspensions and their effects on collagen-induced activation measured. We found that cyclized peptides based on both domains display inhibitory activity. Both domains therefore recognize collagen receptors ($\alpha_2\beta_1$ integrin or/and GPVI) on the surface of platelets. It was surprising, however, that an Ala-substituted, linear peptide based on the Cys-rich region of Atrolysin A, aggregated washed platelets, in a dose-response manner. Experiments with function-blocking antibodies showed that this agonist peptide did not require platelet $\alpha_2\beta_1$ integrin, $\alpha_{IIb}\beta_3$ integrin or GPIb to induce this effect. On interaction of this peptide with platelets, a sustained strong increase in protein tyrosine phosphorylation was observed, together with activation of mitogen-activated protein kinase (MAPK), ERK2. The protein tyrosine phosphorylation induced by the agonist peptide was inhibited by the Src kinase specific inhibitor, PP1. Although the mechanism of action of this peptide on platelets is not fully understood, the unexplored cysteine-rich region of the venom metalloproteinases may provide useful tools for the investigation of platelet surface components that are involved in platelet activation.

P17

HOPLONEMERTINE PYRIDYL ALKALOIDS: INTERACTIONS WITH NICOTINIC RECEPTORS.

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Hoplonemertine worms utilize a variety of pyridyl alkaloids to capture their prey and to defend themselves against predators. Many of these compounds interact with nicotinic receptors. We have recently identified some new structures and actions for pyridyls found in *Amphiporus angulatus, Aa*, a species found in northern Pacific and Atlantic intertidal habitats. Two trace constituents, anabaseine and anabasine, are potent agonists on a wide variety of nicotinic receptors in invertebrates as well as vertebrates. 2,3'-bipyridyl, an unionized alkaloid which is the most abundant *Aa* neurotoxin, is only a weak agonist at vertebrate neuronal and muscular nicotinic receptors. However, it is about 4X more toxic on crustaceans than anabaseine. We have examined the binding of various *Aa* pyridyls to spiny lobster and horseshoe crab ganglionic nicotinic receptors. Both 2,3'-bipyridyl and anabaseine displace alpha-bungarotoxin from putative nicotinic receptors. Crustacean ganglionic nicotinic receptors were also characterized using radiolabelled cytisine and epibatidine as radioligands. Apparently a sub-population of nicotinic receptors binding the snake toxin does not require that the nicotinic ligand bears a positive charge for receptor occupation. (Supported by Florida Sea Grant R/LR-MB-9)

P18

AMPHIPHILIC PEPTIDES FROM SOLITARY WASP VENOMS.

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Solitary wasp venoms are a rich source of bioactive molecules, such as acylpolyamines, biogenic amines, and peptides. However, our most recent studies indicated that these venoms may also contain amphiphilic α-helical peptides presenting antimicrobial activities.

Solitary wasps inject their venom into arthropod prey and paralyze them in order to feed their larvae. Therefore, these venoms must contain neurotoxins acting on nervous systems. However, our recent studies have demonstrated that these venoms also contain various bioactive components in addition to neurotoxins. We have isolated eumenine mastoparan-AF (EMP-AF) from the venom of the eumenine solitary wasp *Antherhynchium flavomarginatum micado*. The primary sequence of EMP-AF was similar to that of mastoparan, a mast cell degranulating peptide from a hornet venom. In fact EMP-AF presented a high degranulation activity of rat peritoneal mast cell and a significant hemolysis of human erythrocytes.

Further characterization of EMP-AF demonstrated that this peptide exhibited a potent inhibitory activity both against Gram-positive and Gram-negative bacteria, with significant selectivity to Gram-positive ones. This is the first description of an antimicrobial peptide in solitary wasp venom. Other peptides isolated from different species of solitary wasps having similar properties to EMP-AF will also be presented.

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P19

THE IMPORTANCE OF HYDROPHOBIC RESIDUES IN ERISTOSTATIN'S BINDING TO MELANOMA CELLS.

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The disintegrin eristostatin inhibits melanoma metastasis in a mouse model. This is thought to occur when the disintegrin binds to an integrin on the cell. Disintegrins containing the sequence RGDWN, such as eristostatin, bind to the integrin $\alpha IIb\beta 3$ with a higher affinity than to $\alpha v\beta 3$ or $\alpha 5\beta 1$. For echistatin, a structurally similar disintegrin containing the sequence RGDDM, the opposite is true. In our study, we investigated if placing a hydrophobic residue at position RGDXX would alter eristostatin's affinity for the yet unidentified receptor with which it associates. We expressed recombinant eristostatin, echistatin, and four mutations targeting amino acids in the RGD loop. Human 1205LU, WM164, and C8161 melanoma cells were incubated in eight-chambered slides in the presence of fluorescent-labeled eristostatin ± unlabeled disintegrin. Human MV3 melanoma cells were incubated with fluorescent-labeled anti-$\alpha 4$ ± unlabeled disintegrin. The cells were observed by confocal microscopy. For 1205LU, WM164, and C8161 cells, all unlabeled proteins with methionine or leucine at position RGDXX inhibited labeled eristostatin's binding more strongly than unlabeled eristostatin did. Unlabeled eristostatin did not inhibit the binding of anti-$\alpha 4$ to MV3 cells when asparagine at position 31 was replaced with methionine. These results suggest that asparagine is critical for eristostatin's binding to MV3 cells but not to the other three cells, and hydrophobic residues such as methionine or leucine enhance the ability of the disintegrin to interact with 1205LU, WM164, and C8161 cells.

PRODUCTION IN E. coli OF ANTI-TETANUS HUMAN SINGLE-CHAIN ANTIBODY FRAGMENTS WITH TOXIN-NEUTRALIZING ACTIVITY.

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Anti-tetanus human monoclonal antitoxin preparations are more desirable, because of their less-limited sources and no risk of viral contamination, than current human polyclonal IgG preparations. To obtain economically anti-tetanus human antitoxin preparations of monoclonal origin, we established an expression system in E. coli for single-chain antibody fragment(ScFv)s of anti-tetanus human monoclonal antibody MAb-G6 with very high toxin-neutralizing activity. cDNA fragments encoding the variable regions(K and H) of the light (kappa) and heavy chains of MAb-G6 were cloned from total RNA isolated from hybrid cell line G6 into a vector pCANTAB 5E (Pharmacia) for phage display, so as to be translated in E. coli suppressor strain HB2151 in the following forms : soluble ScFv composed of K and H using (GGGGS)n as linkers (Ln, n:1~3 repeats) between the two regions, from N-terminus to C-terminus in this order (K-Ln-H) and vice versa (H-Ln-K) were produced. In tests for toxin-binding activity by ELISA, induction of the transfected E. coli with 1 mM IPTG at 37°C for 48 h produced ScFv mostly into the culture fluid, while that at 30°C for 3.5 h gave most of ScFv in the cytoplasmic fraction of the sonic extracts of the bacteria and its expression level was ca. 5 times as high as that in the culture fluid. The expression levels(ELISA) of ScFv of H-Ln-K types were remarkably higher than those of ScFv of K-Ln-H types and the level of H-L_1-K type with the shortest linker was the highest. In toxin-neutralization tests in mice, the mixtures of toxin (10 MLD) and ScFv preparations resulted in remarkably delayed death (up to ca. 200 h after injection, 6 times as long as the time until death by toxin alone) in a dose-dependent manner. High doses of ScFv (H-L_1-K type) preparations are now being tested for complete neutralization (rescue).

P21

PHARMACOLOGICAL EVALUATION OF ANTIMYOTOXIC NEW COUMESTANS WITH DIFFERENT PATTERNS OF OXYGENATION.

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As part of a program aimed at synthesizing biologically active flavonoids, some coumestans bearing different patterns of oxygenation at rings A and D were chosen as target molecules, synthesized for the first time and their action as antimyotoxic compared with that observed for wedelolactone (W). Coumestans were prepared by oxidation of pterocarpans and in vitro antimyotoxic activity determined using mouse EDL (Toxicon 32:595, 1994). At 30 μM, all five W analogs antagonized the increase of creatine kinase release induced by *Bothrops jararacussu* venom. Compound 2b inhibited the venom myotoxic activity in a concentration-dependent manner, with a IC50 similar to the one of W (1μM). 2b and wedelolactone were submitted to radioreceptor and enzymatic assays in order to screen for different potential molecular targets. Both W and 2b were relatively potent (IC50 1 mM) for inhibiting rat kidney Na+,K+-ATPase but only W potently inhibited [3H]-flunitrazepam binding to rat brain synaptosomes (IC50 2 mM). As W, 2b inhibits the proteolytic and phospholipase activities of *Bothrops* venom. The newly synthesized 2b is equipotent to W for its antimyotoxic action and inhibition of Na,K-ATPase but much more less potent for binding to benzodiazepine receptors so that it should be less susceptible to produce adverse effects in the central nervous system.

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P22

KALLIKREIN-LIKE AND ANGIOTENSIN-DEGRADING ACTIVITIES OF TWO SERINE PROTEASES ISOLATED FROM DESERT MASSASAUGA (*Sistrurus catenatus edwardsi*) venom.

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This study investigated two serine proteases exhibiting kallikrein-like activity (K1, K2) isolated from venom of the desert massasauga (*Sistrurus catenatus edwardsi*). Venom obtained from *Sistrurus catenatus edwardsi* was fractionated using size exclusion, ion-exchange and high performance liquid chromatographies. The isolated proteases (K1, K2) were both found to be glycoproteins with molecular weights of 26.8 kDa and 27.5 kDa, respectively. Kallikrein-like and thrombin-like activities were measured using N-Bz-Pro-Phe-Arg-pNA and N-Bz-Phe-Val-Arg-pNA, and the enzymes were assessed for angiotensin-degrading activity using HPLC and for fibrinogenolytic activity using SDS-PAGE. Results demonstrate that both K1 and K2 show 30-fold greater specificity for the kallikrein substrate, and both enzymes exhibit angiotensin I-degrading activity. One of these enzymes (K2) also degrades the Bβ subunit of fibrinogen but does not degrade the Aα subunit significantly, while the other (K1) does not significantly affect any of the subunits. These kallikrein-like glycoproteases join a growing number of serine proteases found in viperid and helodermatid venoms that have been reported to exhibit angiotensin-degrading activity. The presence of enzymes exhibiting multiple activities indicates that numerous sites of prey hemostasis are targeted, and their actions explain the acute hypotension that is a common and immediate manifestation in prey (and often humans) envenomated by rattlesnakes.

P23

ISOLATION AND CHARACTERIZATION OF A PHOSPHOLIPASE FROM THE VENOM OF *Bothrops erythromelas*.

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Phospholipases A_2, esterolytic enzymes that hydrolyze 3-sn-phosphoglycerides at the sn-2 position, are ubiquitous in snake venoms. The various phospholipase A_2 subclasses possess a broad spectrum of pharmacological activities. In this work, a probable phospholipase A_2 (BePL) was purified from *Bothrops erythromelas* venom using molecular exclusion chromatography (Superdex 200) and reverse phase chromatography (Source RPC - C_{18}). BePL represented represented 5.6% of total venom protein and approximately 4.7% of crude venom solid constituents. Enzyme purity was confirmed by SDS PAGE on 15% gels and by immunoblotting. The molecular weight of BePL, according to SDS PAGE was 13 kD. The purified enzyme presents roughly 8-fold higher phospholipase activity against egg yolk suspensions than does the crude venom. BePL is most active in alkaline solution (pH 9.0) and displays a thermal optimum of 60° C. BePL does not cause hemorrhage or *in vitro* coagulation, nor does it have anticoagulant activity *in vivo*. BePL increase capillary permeability in mice, as indicated by the release of Evans Blue dye into interstitial tissues; however, it does not cause edema in rats. BePL is non-lethal to mice at doses as high as 30 µg/g *i.p.*

P24

A DERMASEPTIN-RELATED PEPTIDE ISOLATED FROM THE SKIN OF THE FROG *Leptodactylus ocellatus.*

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The exudate of frog skin contains an array of gene-encoded peptides with diverse biological functions. Looking at the physiological and evolutionary aspects, the main functions attributed to this repertoire of peptides are preventing microbial infection, assisting wound healing and helping to avoid predation. In this report, we describe the purification and partial characterization of a hemolytic peptide obtained from *Leptodactylus ocellatus* cutaneous secretion. Adult specimens of *Leptodactylus ocellatus* were collected in Distrito Federal region and maintained in captivity. Cutaneous secretion was obtained by mild electrical stimulation and lyophilized. The dried secretion (5.0mg) was dissolved in 0.1% (v/v) TFA/water and loaded on a C_8 reversed-phase column. Elution was performed using a linear gradient of acetonitrile, at a flow rate of 0.8 mL/min. The absorbance was monitored at 216 nm. Fractions were manually collected, lyophilized and tested for hemolytic activity. One hemolytic fraction was individually rechromatographed using a C_{18} reversed-phase column, yielding a symmetric peak. This homogenous fraction was submitted to automated Edman degradation resulting in a partial sequence up to the 24th residue. A search using non-redundant database by BLASTP revealed high homology to amphibian antimicrobial peptides included in the dermaseptin family.

P25

SELECTIVE NEUROMUSCULAR BLOCKING EFFECTS OF CANDOXIN (A NOVEL THREE-FINGER α-NEUROTOXIN FROM THE MALAYAN KRAIT) IN ANAESTHETIZED RATS.

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We have recently isolated a novel three-finger α-neurotoxin, candoxin (MW 7334.6), from the venom of the Malayan krait *Bungarus candidus* that produced selective and reversible postsynaptic neuromuscular blockade in isolated tissue preparations. We have now investigated the effects of candoxin on neuromuscular transmission and cardiovascular parameters in anaesthetized rats. Candoxin (0.04 - 0.14 μmoles/kg) produced rapid, dose-dependent neuromuscular blockade in the soleus muscle of the rat that reversed spontaneously or by a bolus infusion of the anticholinesterase neostigmine (40 μg/kg). At doses of up to 0.42 μmoles/kg (3 mg/kg), candoxin did not have a significant effect on the arterial blood pressure, heart rate, respiratory rate and electrocardiogram of the anaesthetized rat. On a molar basis, candoxin was ~10 fold less potent than erabutoxin b or α-cobratoxin in producing neuromuscular blockade. However, the neuromuscular blockade produced by erabutoxin b and α-cobratoxin was irreversible even with high doses of neostigmine (2 x 100 μg/kg bolus doses). Our results show that like the clinically used muscle relaxant d-tubocurarine, candoxin also produces rapid and reversible neuromuscular blockade *in vivo* with no obvious effects on the cardiovascular system.

P26

NEUTRALIZING ABILITY AGAINST THE LETHAL EFFECT OF *Lachesis muta, Crotalus durissus terrificus* AND *Micrurus mipartitus* VENOMS, BY EXTRACTS OF PLANTS USED IN ANTIOQUIA AND CHOCÓ, COLOMBIA.

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More than 700 plants have been reported in the world as used by traditional medicine against snakebites. Recently, Otero et al. reported eight plants: *Citrus limon, Pleopeltis percussa, Brownea rosademonte, Heliconia curtispatha, Trichomanes elegans, Tabebuia rosea, Bixa orellana and Renealmia alpinia,* whose ethanolic extracts inhibited in vitro the lethal effect of *Bothrops atrox* venom from Antioquia and Chocó. In this work, we studied the neutralizing ability of these plants against the lethal effect of *L. muta, M. mipartitus and C.d.terrificus* venoms. Sublethal doses of every extract were preincubated at 37 °C during 30 min with 1.5 LD_{50} (182, 13.5, 2.7 µg / mouse of *L.muta, M. mipartitus and C.d. terrificus* venoms respectively) and then, the mixture dissolved in 0.5 ml PBS pH 7.2 was injected i.p. to groups of 10 mice (18-20g body weight). In all the experiments, a control group of 10 mice received the venom alone. The survival rate was registered during 48 - 72 hr. All the extracts demonstrated 100% of neutralization against 1.5 LD_{50} *L. muta* venom. Nevertheless, only seven of them neutralized the lethal effect of *C.d.terrificus and M.mipartitus* venoms. The highest potency was that of *B. rosademonte* (Effective doses 100%= 500,125,15.6 µg/mouse for the three venoms, respectively). The extract of *C. limon* did not neutralize the neurotoxic venoms, even at doses up to 8 mg/mouse. The extract of *P. percussa* only neutralized partially the *M. mipartitus* venom, with 80% of survival rate within 72h.

APOPTOSIS INDUCED BY [LYS^{49}] PHOSPHOLIPASE A_2 ISOZYMES FROM *Trimeresurus flavoviridis* (HABU SNAKE).

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The venom of *Trimeresurus flavoviridis* (Habu snake) is a complex mixture of a variety of biologically active proteins. In order to identify the factors that are responsible for cell death, we isolated basic proteins I and II (BPI and BPII) which can induce cell death in human promyelocytic leukemia cells (HL-60). BPI and BPII are isozymes of lipolytically highly active [Asp^{49}] phospholipase A_2 (PLA_2), and classified as [Lys^{49}]PLA_2 . They have only 1-2 % lipolytic activity of [Asp^{49}]PLA_2. Exposure of HL-60 cells to increasing concentration of BPI or BPII caused apoptosis as evidenced by: (1) morphological changes in light microscopy, (2) nuclear fragmentation indicated by propidium iodide staining in flow cytometry, and (3) nuclear condensation detected with Hoechst 33258 in fluorescence microscopy. By contrast, [Asp^{49}]PLA_2 failed to induce cell death in HL-60 cells in spite of higher doses. Enzymatically inactive His-48-*p*-bromophenacylated BPII, however caused cell death in HL-60 cells. These observations indicated that BPII-induced apoptosis is independent of PLA_2 enzymatic activity. In order to determine the possible involvement of caspase 3 or 6 dependent pathways in BPI or BPII induced apoptosis, HL-60 cells were cultured with or without caspase 3 or 6 inhibitor. Neither caspase 3 nor 6 inhibitor rescued the cells from BPI or BPII-induced apoptosis. No activation of intrinsic caspase 3 or 6 activity was found in BPII-treated HL-60 cells. Poly (ADP-ribose) polymerase known as a substrate of caspase family enzymes remained unchanged in BPII treated HL-60 cells. All the data together, it is said that BPI or BPII-induced apoptosis in HL-60 cells is independent of PLA_2 enzymatic activity and is not associated with caspase apoptotic cascade.

INHIBITORY ACTIVITY OF PYROGLUTAMIL AMINOPEPTIDASE II (TRH-DEGRADING ECTOENZYME) IN EXTRACTS OF MARINE ORGANISMS.

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TRH is a neuropeptide widely distributed in brain. Its neuroendocrine role is well established in the control of the biosynthesis and release of thyrotropin and prolactin (PRL) and in extrahypothalamic areas also acts as a neurotransmitter and/or neuromodulator. Once released into the synaptic cleft, TRH is inactivated by a specific neuronal metaloenzyme, TRH-degrading ectoenzyme also known as pyroglutamil aminopeptidase II (PPII, EC 3.4.19.6). PPII is a type II cell surface peptidase, located on synaptosomal and adenohypophysial membranes and is the most specific neuropeptidase described up to now. PPII inhibitors may be used to enhance the therapeutic actions of TRH and to investigate the functions of TRH and PPII in the central nervous system. Marine organisms are an important source of bioactive polypeptides with different activities such as cytolysins, potassium-channel modulators and proteinases inhibitors of all mechanistic classes. The present contribution describes the screening of pyroglutamil aminopeptidase II inhibitory activity in extracts of 23 marine species belonging to the Phylla Chordata, Coelenterata, Mollusca, Annelide, Echinodermata, Algae, Poriphera, Cnidaria. Animals were collected along the northern coast of Havana, Cuba. The aqueous extracts were prepared by homogenisation of the whole animal bodies and freeze-dried. Inhibitory Activity of PPII was found in 5 species (Chordata, Poriphera, Mollusca, Annelida). The molecule with inhibitory activity in *Hermodice carunculata* extracts was purified to homogeneity by treatment with 2.5% TCA followed by an anionic exchange chromatography on DEAE-Sephacel and molecular exclusion on Sephadex G-25. Mass spectrometry (FABS) indicates a Molecular Weight of 750 Da. Analysis of its aminoacid composition revealed the presence of Glu, Asp, Ser, Gly and Thr . The inhibitor is stable at neutral pH and temperatures below 50° C for at least 24 hours. The study of the specificity of its inhibitory activity against peptidases belonging to all mechanistic classes (Serine: trypsin, chymotrypsin, elastase, DAP IV, thrombin. Aspartic: pepsin. Cystein: bromelain, papain, PPI. Metallo: collagenase, gellatinase, carboxipeptidases A and B, angiotensin-converting enzyme and aminopeptidase M) indicates that the inhibitor is highly specific in its interaction with PPII because it failed to inhibit all enzymes tested. In primary cultures of adenohypophyseal cells addition of 1, 3 or 5 ug of the inhibitor resulted in PRL secretion of 1.7, 2.2 and 4.1 times that of controls. TRH administration induced a PRL secretion 3.5 times that of the control and in the presence of both molecules, the PRL discharge was more than 10 times the control release indicating a synergism between TRH and HcPI.

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P29

QUICK METHOD OF MEASURING THE EFFECTS OF SNAKE VENOM ON THE HUMAN CLOTTING CASCADE AND NEUTRALIZATION OF THE VENOM.

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There are 44 subspecies of poisonous snakes in the United States and the venoms are different. The variable and complex nature of snake venoms creates medical emergencies in which the physician encounters different clinical symptoms with each new snakebite victim. One of the major concerns of the physician is the effect of snake venom on the clotting cascade. In this study, a Sonoclot analyzer was used to screen venom fractions that affected the coagulation cascade in human blood. Each fraction was analyzed for initial onset of coagulation (onset time), clot rate, and platelet function (clot retraction). Two commercial antivenins and four animal sera were used to neutralize venom fractions that interfered with the coagulation cascade in human blood. A Sonoclot analyzer was used as a rapid screening method (15 min) for molecules that affected the coagulation process.

P30

OCCURRENCE OF TETRODOTOXIN (TTX) AND DERIVATIVES IN THE BRACHYCEPHALID FROG *Brachycephalus pernix*.

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Specimens of *B. pernix* were collected in Curitiba, Santa Catarina, a region of Brazilian Atlantic Forest. Whole body, skin and liver were separately homogenized in MeOH/acetic acid 4%, and stored at 4° C. The extracts were evaporated, dissolved in deionized water, and the lethal potencies estimated by i.p. injection in white mice (19-20g). One mouse unit (MU, 0.22ug of TTX) was standardized as the amount of extract that kills the animal after 30 minutes of injection. The extracts were semipurificated using an exchange ionic column (Amberlite CG-50), treated with active charcoal (Norit-A), and analyzed by HPLC on a reverse phase column with post column fluorimetric reaction system. The extracts and fractions were also submitted to MALDI-TOF mass spectrometry. The toxicity of the whole body was 345.21 MU/g, tissues toxicity assays showed 980.98 MU/g and 345.21 MU/g respectively for skin and liver. HPLC analysis and mass spectra demonstrated the occurrence of TTX, tetrodonic acid, 4,9 anhydroTTX and 4-*epi*TTX. The mass spectra (MALDI-TOF) of the semipurificated skin extract confirm the occurrence of TTX exhibiting mass at 320 Da assignable to TTX. The present work confirms, by HPLC and mass spectra, *Brachycephalus pernix* as the second species in the brachycephalid family which contains TTX.

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AMINO ACID SEQUENCING AND HISTOPATHOLOGICAL STUDIES OF BUCARIN, A HELVEPRIN FROM *Bungarus candidus* (MALAYAN KRAIT) VENOM.

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Snake venom is a mixture of pharmacologically active peptides and proteins that fall into a few families. We recently purified and determined the N-terminal sequence of a number of proteins that belong to a new venom protein family called helveprins (**hel**othermine-related **v**enom **p**rotei**ns**). These proteins (MWs of 19-25 kD) are homologous to helothermine from the venom of the Mexican-beaded lizard *Heloderma horridum horridum,* as well as mammalian testis-specific cysteine-rich proteins. Here we report the purification and histopathology of bucarin. The protein was purified by a two-step method (gel filtration followed by reverse-phase HPLC). The molecular mass was 23,881. Amino terminal sequencing of native bucarin yielded the first 55 residues. This segment has 58% identity and 75% homology with helothermine. Native bucarin was digested with endoproteinase Lys C and the resultant peptides separated by RP-HPLC. These peptides were sequenced either directly or after reduction and pyridylethylation. Using this strategy, several internal sequence peptides have been determined. When bucarin was injected (intraperitoneally;10 mg/kg) into mice, no symptoms were observed. All vital organs (brain, heart, kidney, lung and liver) in the mice injected with the toxin appeared structurally normal. We are currently investigating other potential biological activities of bucarin in the rat model.

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PARTIAL PURIFICATION OF A FIBRINOLYTIC ENZYME FROM POOLED VENOM OF *Agkistodon piscivorus leucostoma.*

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Agkistrodon piscivorus leucostoma, known as the water moccasin or the western cottonmouth, is partly an aquatic snake, living in or near water and feeding mainly on fish and amphibians. The cottonmouth resides in the Southeastern United States and is aggressive when threatened. Then venom contains metalloproteinases that are hemorrhagic and fibrinolytic and both have important biomedical applications. One particular enzyme is a fibrinolytic enzyme that could potentially be used in the treatment of strokes and heart attacks. The purpose of this study was to isolate a fibrinolytic enzyme from *A. p. leucostoma* venom by high performance liquid chromatography (HPLC) with a DEAE column and measure fibrinolytic activity. Electrophoretic titration was used to determine the optimal conditions for HPLC separation. Crude venom was initially eluded with 0.02M Tris-HCl buffer pH 8.0 and hemorrhagic activity was found in fractions 6-8. Fraction 5 had the highest fibrinolytic activity and was further purified by refractionation at a lower pH. An electrophoretic titration of fraction 5 showed the optimal pH for further separation was 6.5. SDS-PAGE electrophoresis revealed seven bands with a molecular range from 14-43 kDa. When fraction 5 was refractionated, none of the fractions exhibited hemorrhagic activity. In the refractionation, fractions 2-6 all contained fibrinolytic activity with fraction 4 having the highest specificfibrinolytic activity (1.917 mm/μg). Electrophoretic titration of fraction 4 revealed 5 bands and optimal pH of 7 for further separation. Fraction 4 will be further purified and monoclonal antibodies will be produced. Gelatinase activity was present in fibrinolytic fractions 2-4. Fraction 2 activated plasminogen, while the other two did not initially activate the plasminogen. Antifibrinolytic assay results revealed that *Didelphis virginiana* serum inhibited fibrinolytic activity in fractions 3 and 4, but did not neutralize fraction 2.

PURIFICATION AND PARTIAL CHARACTERIZATION OF A CARBOXYPEPTIDASE LIKE PROTEASE AND ITS INHIBITOR FROM *Sabellastarte magnifica* (Annelida).

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The mammalian carboxypeptidases (CP) represent an important and diverse class of enzymes with different digestive and regulatory functions. Consequently, their inhibitors are molecules with potential effectiveness in Biotechnology and Biomedicine. The variety of inhibitors (PI) of CP found in nature is somewhat limited, as compared to those of other proteases. The availability of new inhibitors will contribute to CP structure-function advances and to the development of new potential drugs. Among all possibilities of natural sources of PI, one of the most attractive and unexplored is the marine fauna, specially invertebrates. From an screening of inhibitory activity against CPA in more than 50 Cuban marine invertebrates, the annelide *Sabellastarte magnifica* was the most promising. Extracts prepared from the animal tentacles crown revealed a high inhibitory activity against CPA. The inhibitor (SmCI) was purified combining heat treatment, affinity chromatography on CPA immobilized on glyoxyl Agarose and RP-HPLC (C-8). Its molecular mass is 19 kDa (MALDI-TOF) and the N-terminal sequence has not revealed homology with other CPA inhibitors described. It is active, not only against pancreatic CPA (Ki 10^{-10} M), and other CP, like pancreatic and plasma CPB, but also against trypsin. On the other hand, a CPA like protease (SmCPA) was purified from the animal body extracts. It is a protease of 34 kDa, and high homology (N-terminal sequence) with other CP. SmCPA is inhibited by different specific CP inhibitors.

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DETERMINATION OF HEMORRHAGIC ACTIVITY OF *Bothrops* VENOMS.

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Hemorrhagic activity of *Bothrops (B.) alternatus, B. jararaca, B. moojeni* and *B. neuwiedii* venoms from specimens captured in Argentina was compared in mice by measuring the average diameters, the weight and the amount of hemoglobin extracted from the excised hemorrhagic haloes as a function of the venom dose. Only the measurement of the amount of hemoglobin extracted allowed to detect differences in potency of the hemorrhagic activity. The plots of amount of hemoglobin extracted as a function of the weight of the excised hemorrhagic haloes resulted straight lines passing through the origin, with a slope (average amount of hemoglobin extracted per gram of hemorrhagic halo) characteristic for each venom, proportional to the potency of its hemorrhagic activity and independent of the venom dose. In a similar study performed on rats it was found that the MHD were 19 to 56 fold higher than the MHD in mice. Measurement of the amount of hemoglobin extracted from the hemorrhagic haloes showed significant differences in potency. The amount of hemoglobin extracted per gram of hemorrhagic halo was again a constant, characteristic for each venom and independent of the venom dose, with values similar to those obtained with mice. Inactivation by preincubation at low pH values or the presence of EDTA as well as inactivation of proteolytic activity on gelatin and decrease in lethal potency, exhibited a high positive correlation when hemorrhagic activity was determined by measurement of the amount of hemoglobin extracted from the hemorrhagic haloes.

EXPERIMENTAL ANTI-*Loxosceles laeta* ("VIOLINIST") ANTIVENOM.

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Bites by *Loxosceles (L.) laeta* spiders are a public health problem in Argentina. The specific treatment is the use of antivenom (AV) at the first stages of envenomation. The production of AV is difficult due to the low venom yield by these spiders and the difficulties in maintaining a large breeder of *Loxosceles*.We developed an experimental AV for *L. laeta*, using as immunogen venom glands homogenates from spiders captured in Argentina. A reduced immunization protocol was employed which required 1.0 mg antigen/horse. Hiperimmune plasma from horses was fractionated by saline precipitation and treated with pepsin to obtain $F(ab')H_2$ fragments. ELISA and Westernblot were used to compare the immunochemical reactivity with that of other anti-*Loxosceles* antivenoms (AVs) available in Argentina. The neutralizing capacity of the lethal potency (mice) and necrotizing activity (rabbits) were determined. Our results showed that the heterologous AV was effective in terms of neutralization of lethality and necrosis, however the homologous AVs (the experimental and the therapeutically used) showed a higher immunochemical reactivity and neutralizing capacity against venom induced lethality and necrosis. The SDS-PAGE analysis of the experimental AV showed $F(ab')H_2$ fragments while other Avs showed bands of entire IgG plus other plasma proteins.

DNAse ACTIVITY OF SNAKE VENOMS.

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We studied the hydrolytic activity on DNA of venoms from *Bothrops (B.) alternatus, B. neuwiedii, B. ammodytoides, B. jararaca, B. jararacussu, B. moojeni, B. asper, Crotalus (C.) durissus (d.) terrificus, C. (d.) durissus, C. basiliscus, C. scutulatus, Athropoides (At.) nummifer, Agkistrodon (Ag.) billineatus, Naja (N.) siamensis, Bitis gabonica* and *Trimeresurus (T.) okinawensis*. The activity was studied on agarose gels containing DNA and ethidium bromide and it was determined as the diameters of the hydrolytic haloes as visualized by U.V. illumination. The higher activity was observed using venoms from *B. neuwiedii, B. jararacussu, B. jararaca, B. moojeni, B. asper and N. siamensis*. The venoms from *B. alternatus, C.d. terrificus, C.d. durissus, C. scutulatus, At. nummifer, Ag. billineatus and Bitis gabonica* showed lower activity than the first group. Venoms from *B. ammodytoides* and *T. okinawensis* showed very low activity. The *Bothrops* venoms (excepting *B. ammodytoides*) showed higher activity. In the zymograms after SDS-PAGE it was observed that only the venoms with higher activity on radial hydrolysis produced hydrolytic bands. The molecular weight in which the bands were located ranged in the order of 20-60 kDa and the hydrolytic activity was fully inhibited by EDTA. When *B. neuwiedi* or *N. siamensis* venoms were preincubated with antibothropic or anti-*N.siamensis* antivenom it was observed homologous and heterologous inhibition of the DNAse activity in a dose dependent manner.

SCORPIONISM BY *Tityus trivittatus* IN ARGENTINA.

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The scorpion *Tityus trivittatus* is the only one specie reported dangerous to human in Argentina. It can be found in large numbers in the North West, and it is also found in the Center and East, where it had becoming adapted to a synanthropic life. Because local data about the clinical picture of accidents by this scorpion are scarce, and from rather limited regions, the objective of this work was to characterize clinically this envenomations. There were studied 461 stories from stung people who received specific treatment with antivenom. The most frequent signs and symptoms at the site of bite were local pain (90%), edema (29%), burning (29%), erythema (22%), and paresthesia (10%) and the presence of cramps (4%). Some people showed headache (12%) and excitation (9%). The signs involving the cardiovascular system were paleness and tachycardia (20%). Hypotension, arrhytmia, dyspnea, cyanosis, bradychardia and precordial pain were not frequent (<5%). This symptoms suggest an adrenergic autonomic disturb, involving specially sympathetic system. The finding of paleness (~20%) and hypothermia (5%) strengths this possibility. In the other hand, there were observed vomiting (27%), sweat (9%) and sickness (5%), signs which indicate cholinergic stimulation, involving parasymphathetic system. It was observed that the systemic signs are more frequent in the group of 0-10 years ($p<0.01$) whereas no differences were observed ($p>0.1$) among the other groups (11-20, 21-50 and >$50). The mortality rate observed was around 6.5 over 1000.

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SOME STUDIES ON BEE VENOMS (*Apis mellifera mellifera*) FROM BUENOS AIRES.

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Protein composition and hemolytic, myotoxic activities and lethal potencies of venoms from bee from different regions of Buenos Aires, were compared. No major differences were observed in the SDS-PAGE profiles which showed stained bands with molecular weights lower than 50 kDa. Lethal potencies assayed on mice (10-15µg/g, $p<0.05$) showed positive correlation with hemolytic activity (r^2 0.74). No differences were found ($p>0.05$) among direct or total (direct plus indirect) hemolysis in the samples tested. However the EC50 for total hemolysis (6-12µg) were 2-3 fold higher ($p<0.05$) than those for direct hemolysis (2.5-4.0µg). Equine erythrocytes are to be more sensitive to venom induced direct and indirect hemolysis than human, sheep or rabbit erytrocytes. After the i.m. injection of bee venom to mice, the levels of free hemoglobin and CPK were higher ($p<0.05$) than in control mice. No proteolytic or coagulant activities could be detected in the venom samples studies. Serum from horses immunized with bee venom was able to neutralize the hemolysis produced by 50µg of venom (DEH_{50} 0.75±0.03 mg of antivenom, around 15µg of IgG per µg of venom).

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ANTIEDEMATOUS, CYTOTOXIC AND NEUROTOXIC ACTIVITIES IN EXTRACTS OF THE SEAWEED *Galaxaura marginata* (RHODOPHYTA, NEMALIALES).

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An antiedematous, cytotoxic and neurotoxic activities of substances extracted from *Galaxaura marginata* was investigated trough pharmacological assays, using seaweeds collected at São Sebastião channel (45°25' W;26°49'S) in the North litoral of São Paulo State, Brazil. Apolar substances inhibited the local edema in the mouse ear produced by *Croton* oil, inhibited of the embryonic development of sea urchin eggs and induced hemolysis in mouse erythrocytes in low concentrations. The polar fractions (H3 and H4) inhibited the embryonic development of sea urchin, at similar concentrations, but only H3 is able to induces hemolysis. The neurotoxic effects of polar fraction H4 was lethal in high concentration when injected intraperitoneally in mouse. The previous symptoms preceding death was characterized by loss of the motile control and disorientation, extensor muscle contraction (H3) and H4 induced a total flaccidity of body and progressive reduction of body temperature . The H4 was filtered through membrane of 1000 Da MW cutoff, and was injected intracraneally in the right ventricule of rat brain, and it produces the same symptoms preceding death, as described in mouse, demonstrating that H4 crosses the blood brain barrier. Thus, we can say that *G. marginata* have apolar substances with antiedematous and cytotoxic activities and polar substances with similar activities in the embryonic development of sea urchin with different lethal activity. Moreover, H4 fraction crosses de blood brain barrier.

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SKIN SECRETION OF THE CAECILIAN *Siphonops paulensis* FORMS VOLTAGE-DEPENDENT IONIC CHANNELS IN LIPID MEMBRANE.

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The effect of the skin secretion of the amphibian *Siphonops paulensis* was investigated by monitoring the changes in conductance of artificial planar lipid bilayers. Skin secretion (SS) was obtained by exposure of the animals to ether saturated air. Artificial lipid bilayers were obtained by spreading a solution of azolectin over an aperture of a Delrin cup inserted into a cut-away PVC block, delimiting two compartments, *cis*-side and *trans*-side. The first one was filled with 150mM KCl, 15mM NaCl, 100μM $CaCl_2$, 10mM HEPES-TRIS (pH 7), and the *trans*-side with 150mM NaCl, 15mM KCl, 100μM $CaCl_2$, 10mM HEPES-TRIS (pH 7). The SS was added to the *cis*-side, and changes in the bilayer conductance were monitored using an Axopatch 200A amplifier, and recorded in digital form on video tape employing an analog-digital converter. Data were analysed using pCLAMP-6. In 9 of 12 experiments, the addition of the SS to lipid bilayers displayed voltage dependent channels with average unitary conductance of 230pS, rather than non-specific changes in the bilayer conductance. These channels were not sensitive to SITS or TEA. The SS has an hemolysin whose mechanism of lysis probably involves its insertion in the membrane, and the channel formation. The experimental protocol used does not permit to specify the characteristics of the channels.

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PRIMARY STRUCTURES OF FOUR TRYPSIN INHIBITOR E HOMOLOGS FROM VENOM OF *Dendroaspis angusticeps*. STRUCTURE-FUNCTION COMPARISONS WITH OTHER DENDROTOXIN HOMOLOGS.

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Four trypsin inhibitor homologs from the venom of *Dendroaspis angusticeps* were characterized using a combination of gel filtration, cation exchange, reverse-phase liquid chromatography, Edman degradation and mass spectrometry. The four toxins comprise two 57 residue and two 59 residue isoforms. The long toxins possess a Lys-Gln N-terminal extension lacked by the short toxins. The only other structural difference is an Arg/His replacement at position 55. The long, Arg_{55} variant is identical to trypsin inhibitor E from the venom of *Dendroaspis polylepis*. The name ε-dendrotoxin is suggested, so as to follow the nomenclature of Benishin et al. (1988, Mol. Pharmacol. 34, 152-159). Among snake venom protease inhibitors, the ε-dendrotoxins are structurally most like the δ-dendrotoxins, with which they share only 64% of their residues. In addition, the ε-dendrotoxins display hydropathy profiles more like those of the α- and δ-dendrotoxins, than those of the trypsin inhibitors from snake venoms. Hydropathy profiles also suggest that the structurally related weak trypsin inhibitor B (Dendrotoxin B) from the venom of *Dendroaspis polylepis* probably interacts in some manner with biological membranes.

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PREY SPECIFICITY, COMPARATIVE LETHALITY AND COMPOSITIONAL DIFFERENCES OF CORAL SNAKE VENOMS.

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Toxicities of crude venoms from 49 coral snake *Micrurus* populations, representing 15 nominal taxa, were examined in both laboratory mice and in native prey animals and compared with data gathered from two non-micrurine elapids and a crotalid, which served as outgroups. These venoms were further compared on the basis of 23 enzymatic activities. Both toxicities and enzymatic activities were analyzed with respect to natural prey preferences, as determined from stomach content analyses and literature reports. Venoms of nearly all *Micrurus* for which prey preferences are known, are more toxic to natural prey than to non-prey species. Except for amphisbaenians, prey are more susceptible to venoms of *Micrurus* that feed upon them, than to venoms of those that eat other organisms. All venoms were more toxic *i.v.* > *i.p.*> *i.m.* Route-specific differences in toxicity are generally greatest for preferred prey species. Cluster analyses of venom enzymatic activities resulted in five clusters, with the fish-eating *M. surinamensis* more distant from other *Micrurus* than even the crotalid, *Bothrops moojeni*. Ophiophagous and amphisbaenian-eating *Micrurus* formed two close subclusters, one allied to the outgroup species *Naja naja* and the other to the fossorial, ophiophagous *Bungarus multicinctus*. Prey preference is shown to be the most important determinant of venom composition in *Micrurus*.

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EVIDENCE FOR A P-III REPROLYSIN (M12B) FAMILY ZINC METALLOPROTEASE IN THE VENOM OF THE BROWN TREESNAKE (*Boiga irregularis*).

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Envenomations by brown treesnakes, especially of infants and young children, are increasing in Guam, but little is known about the components of this rear-fanged snake's venom. Past studies have demonstrated proteolytic activity in this venom, but no proteases have been identified. This study provides evidence for a class III metalloprotease. The 53 kDa nonreduced/ 58 kDa reduced protease was purified by ion-exchange, reversed-phase chromatography and SDS-PAGE. Specific protease activity of the purified protein toward azocasein was 0.7405 ΔA342nm/min/mg protein, which represents four fold increase in activity from the crude venom. N-terminal sequence data demonstrate that this metalloprotease has high homology with members of the M12B subfamily, specifically with the M12 unassigned peptidases. Sequence identity between *Boiga irregularis* and *Atractaspis microlepidota* metalloproteinases was greater than 60%, with over 80% sequence homology. High homology was also seen with the *Naja naja* cobrin peptidase, the metalloproteinase-disintegrin-like protein from *Agkistrodon contortrix*, and the human disintegrin protease, indicating a complex superfamily relationship among these proteases. These data provide the first structural evidence for the presence of reprolysin family proteases in brown treesnake venom. As one other colubrid snake (*Dispholidus typus*) has been shown to possess reprolysin-type proteases, it is probable that the M12B metalloproteases are distributed among all venomous snake families, and complete sequence data will provide greater insight into the evolutionary relationships of higher snakes.

A SPECIFIC MYOTOXIC PROTEIN PURIFIED FROM THE VENOM GLANDS OF THE TOADFISH *Thalassophryne maculosa* GÜNTER (1861).

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We extracted venom from *T. maculosa* by pressing at the base of dorsal and opercular spines. Pools of venom were freeze-dried and maintained at 80°C until used. The average venom production was 3.57 mg protein/animal. Crude venom yielded 3 fractions [retention times (rt): FI: 12.5, FII: 16.7 and FIII: 22.7 min] by molecular exclusion on Protein Pack 125 (Waters) HPLC columns. F III made 54% of the venom. Nine fractions (C4 rt: 8, 8.7, 11, 15.2, 31, 42.1, 45.7, 47.2 and 66 min) were isolated by reverse phase HPLC on C4 (Vydac) columns. Crude venom (1.04 mg/ml) depolarized frog muscle fibers (*Hyla crepitans*) irreversibly from -84.5 (-88, -81) mV (median and confidence interval, n= 20) to -18 (-23.5, -15) mV (n= 24) ($P= 9'10^{-10}$). F III (37 mg/ml), depolarized fibers from 70 (-81.5, -76) mV (n= 20) to 62.5 (-68.5, -56.5) mV (n= 24) ($P= 5'10^{-6}$). Fraction C4 47.2 (50 mg/ml) depolarized the muscles from 87 (-82, -91) mV (n= 33) to 63 (-75.5, -50.5) mV (n= 53) ($P= 3'10^{-7}$). Washing with normal saline completely reversed these effects. The reversibility was slow and partial if ³100 mg/ml were used. Muscles pretreated with 1mM tetrodotoxin (TTX) were depolarized by C4 47.2 (0.1 mg/ml) with 1mM TTX. C4 47.2 (0.1 mg/ml) was devoid of effects on squid (*Doryteuthis plei*) giant axons resting membrane potential. Yet, C4 47.2 increased miniature endplate potential (MEPP) frequency from 180 ± 18 MEPP/min [30 traces lasting 0.4096 s/endplate (EP) from 3 EPs] to 292 ± 17 MEPP/min (30 traces/EP, 6 EPs) ($P = 0.017$, c^2 test).

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CHARACTERIZATION OF THE MAJOR METALLOPROTEASES ISOLATED FROM THE VENOM OF THE DESERT MASSASAUGA RATTLESNAKE, *Sistrurus catenatu edwardsi.*

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The characterization of rattlesnake venoms (family Viperidae) has historically focused on venoms from the larger *Crotalus* species. Consequently, venoms from the pygmy and massasauga rattlesnakes (*Sistrurus*) are poorly known. As with most viperid venoms, snake venom metalloproteases are prevalent components of desert massasauga venom. This study involves the isolation and characterization of the main metalloproteases from the venom of *S.c. edwardsi*, a diminutive species found in the arid plains of Colorado. Venom samples collected from adult male and female snakes (average yield: 6.5 mg) were combined. Isolation procedures included multiple chromatographic steps using size exclusion and ion-exchange columns; activity was followed using azocasein. Venom from *S.c. edwardsi* contains at lease 4 distinct metalloproteases with molecular weights ranging from 62-87 kDa and having activity toward azocasein. The main protease is an acidic protein with an approximate molecular weight of 75 kDa (SDS-PAGE), suggesting that it is a class III metalloprotease. In solution, this metalloprotease is highly labile as evidenced by the rapid degradation of the parent molecule, producing multiple product bands (SDS-PAGE). Characterization of this enzyme includes determination of metal ion content and assays of activity toward fibrinogen, fibronectin, collagen, and oxidized insulin B chain. Venoms from *Sistrurus* species may contain novel metalloproteases, and as with several other viperid metalloproteases, these enzymes likely hold potential for pharmaceutical development as drugs for treating thrombic disorders and cancers.

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COMPARISON OF DISINTEGRIN EXPRESSION BY TWO STRAINS OF *E. coli.*

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Protein expression is an integral part of our laboratory's disintegrin research. Since bacteria are used to produce these proteins, the cell line that can produce the greatest amount of protein is the most desired. Here we compare the expression capabilities of our current *E. coli* strain, BL21 Gold (G), against that of a newly derived strain, BL21 Codon Plus (CP). The protein produced by both cell lines in this experiment was the 7.5 kDa disintegrin eristostatin. Both strains were transformed with a pGEX-KG plasmid that contained the gene for eristostatin. Cells were lysed and protein was isolated using glutathione resin; the protein was cleaved from the resin using thrombin. HPLC profiles showed that the CP cells expressed a higher concentration of eristostatin than the G cells. This was confirmed by protein yields of 23 µg/L of culture (G) and 116 µg/L of culture (CP). Upon purification of the protein, ADP-induced platelet aggregation was used to determine IC50, because eristostatin will bind to platelets via the integrin receptor $\alpha IIb\beta 3$, and inhibit aggregation. The IC50 values for eristostatin from both strains of cells were comparable to each other and to values previously obtained in our lab. Immunoblots using rabbit anti-eristostatin indicate that the protein was present in the thrombin digests from both cell lines. These results suggest that it would be advantageous to use CP cells for further expression of eristostatin and its mutations instead of the G cells.

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THE ROLE OF PHOSPHOLIPASES A_2 IN THE STIMULATION OF NEUTROPHIL MOTILITY BY COBRA VENOMS.

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Envenoming by the cobras *N. naja* and *N. mossambica* is accompanied by inflammation at the bite site with neutrophils (PMN) being the major cellular component of this process. Using collagen-coated surfaces and Transwell membranes, we demonstrate that the venoms stimulate PMN chemokinesis and chemotaxis parallelled by collagenase secretion and an increase in CD11b and CD49b expression. These effects were reproduced by using purified venom PLA_2s, and were much more pronounced than corresponding PMN responses to fMLP. The effects of the venom and of the purified PLA_2s were partially attenuated by PLA_2 inhibitor aristolochic acid and almost completely abolished by PMN pre-treatment with dexamethasone. AnnexinV and inhibitors of collagenases (Ro 31-9790), cyclo-oxygenase (ASA) and lipo-oxygenase (NGDA), all inhibited PMN motility (Dexamethasone > Annexin V > NGDA > aristolochic acid > ASA = Ro31-9790). FACS analysis and confocal microscopy showed that Annexin V affects PMN motility by interfering with binding and rapid endocytosis of the venom PLA_2. These results suggest that venom-induced PMN motility is caused by the sequence of events involving, in the first instance, receptor-mediated, non-enzymatic stimulation of PMN by venom PLA_2. In response to this stimulation, cytosolic PMN PLA_2 becomes activated leading to the production of lysophospholipids and arachidonate metabolites (PAF, TXA2, LTB4), involved in stimulation of PMN degranulation and motility. Venom PLA_2 then interacts with anionic phospholipids exposed on stimulated PMN, become endocytosed, and may itself contribute to intracellular production of chemoattractants responsible for PMN accumulation at the sites of snakebites.

RATTLESNAKE VENOM METALLOPROTEINASE GENOMIC NUCLEOTIDE SEQUENCES AND MOLECULAR MODELS.

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Most rattlesnakes have abundant metalloproteinases in their venom, proteinases responsible for various physiological effects. Utilizing GeneWalker strategy, we determined metalloproteinase nucleotide sequences from genomic DNA of *C. m. molossus* (blacktailed rattlesnake), *C. atrox* (western diamondback rattlesnake) and *C. s. scutulatus* (Mojave rattlesnake). The metalloproteinase nucleotide sequences from all three snakes had a similar sequence organization, consisting of 5'-UTR, zymogen, proteinase, disintegrin, cysteine-rich domain, and 3'-UTR. One intron was identified, which was present in all three sequences and was located within the proteinase domain. Amino acid sequences deduced from the protein coding region were similar and shared highest sequence identity (>70%) in the zymogen and proteinase domains. All three sequences had the conserved Zn^{2+}-binding site HEXXHXXGXXH, but none had RGD in their disintegrin domain. The cysteine rich domain had the consensus subdomain structure of C-X6-C-X4-C-X6-C-X14-C-X12-C, while subdomain b differed in all three sequences. Molecular models of the proteinase domains were made using crystal coordinates of atrolysin c and adamalysin II. All three models were each topologically similar to the known metalloproteinase crystal structures, retaining all conserved structural elements of the metzincin superfamily of proteinases. There were differences, however, in the spatial arrangement of the structural elements of the active site of the metalloproteinases. Molecular models may help relate structure to function among the snake venom metalloproteinases.

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DISCOVERY AND STRUCTURE OF A POTENT AND HIGHLY SPECIFIC BLOCKER OF INSECT CALCIUM CHANNELS.

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New methods of insect control are urgently required due to the evolution of insect resistance to classical chemical pesticides, growing appreciation of the environmental damage caused by many agrochemicals, and increased public concern about the human health risks associated with prolonged insecticide exposure. Promising new approaches include engineering plants and insect-specific viruses to produce insecticidal toxins. Unfortunately, however, there are few well-characterized peptide/protein toxins that lend themselves to these genomic approaches. Spider venoms can be viewed as pre-optimized combinatorial libraries of insecticidal peptides, and therefore we have been exploiting these venoms in the search for *insect-specific* toxins suitable for engineering into plants and insect viruses [1,2]. Here we describe a new family of insecticidal neurotoxins isolated by screening the venom of the lethal Australian funnel-web spider *Hadronyche versuta*. These toxins appear to be the most potent blockers of insect voltage-gated calcium channels reported to date. These toxins display exceptional phylogenetic specificity, with greater than 50,000-fold preference for insect versus vertebrate calcium channels. The structure of one of the toxins reveals a highly structured, disulfide-rich core and a structurally disordered C-terminal extension that is essential for channel blocking activity. Weak structure/function homology with ω-agatoxin-IVA/B, the prototypic inhibitor of vertebrate P-type calcium channels, suggests that these two toxin families might share a similar mechanism of action despite their vastly different phylogenetic specificities.

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Erratum and *Addendum*

Abstract book

7th Symposium: Pan-American Section of the International Society on Toxinology on Animal, Plant and Microbial Toxins

TALK

ABSTRACTS

PROTEIN ENGINEERING AND THE EVOLUTION OF A VACCINE.

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Throughout the development of a biopharmaceutical, emphasis must be placed on the quality attributes of the product. Quality, safety and effectiveness must be designed and built into the product. Quality cannot be tested or inspected into a finished product. Each phase of the manufacturing process must be controlled, so as to maximize the probability that the finished product will meet all quality and design specifications. Title 21 of U.S. CODE OF FEDERAL REGULATIONS ("21 CFR") states the purpose of Good Manufacturing Practices (GMPs) is "to assure that all pharmaceutical, biologic, diagnostic, and medical device products meet all of the requirements of the Federal Food, Drug, and Cosmetic Act as to safety, and efficacy and have the identity and strength to meet the quality and purity characteristics which they purport to have". This presentation will examine the development of a vaccine candidate against a protein toxin, noting where regulations and science have collided, and how the use of protein engineering technologies have been used to harmonize the two.

POSTER

ABSTRACTS

PHARMACOLOGICAL EVALUATION OF ANTIMYOTOXIC NEW COUMESTANS WITH DIFFERENT PATTERNS OF OXYGENATION.

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As part of a program aimed at synthesizing biologically active flavonoids, some coumestans bearing different patterns of oxygenation at rings A and D were chosen as target molecules, synthesized for the first time and their action as antimyotoxic compared with that observed for wedelolactone (W). Coumestans were prepared by oxidation of pterocarpans and in vitro antimyotoxic activity determined using mouse EDL (Toxicon 32:595, 1994). At 30 μM, all five W analogs antagonized the increase of creatine kinase release induced by *Bothrops jararacussu* venom. Compound 2b inhibited the venom myotoxic activity in a concentration-dependent manner, with a IC50 similar to the one of W (1μM). 2b and wedelolactone were submitted to radioreceptor and enzymatic assays in order to screen for different potential molecular targets. Both W and 2b were relatively potent (IC50 1 μM) for inhibiting rat kidney Na+,K+-ATPase but only W potently inhibited [3H]-flunitrazepam binding to rat brain synaptosomes (IC50 2 μM). As W, 2b inhibits the proteolytic and phospholipase activities of *Bothrops* venom. The newly synthesized 2b is equipotent to W for its antimyotoxic action and inhibition of Na,K-ATPase but much more less potent for binding to benzodiazepine receptors so that it should be less susceptible to produce adverse effects in the central nervous system.

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BIOLOGICAL PROPERTIES OF HEMOLYMPH AND OVA EXTRACT FROM THE BLACK WIDOW SPIDER (*Latrodectus tredecimgutattus*).

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Black widow spiders (genus *Latrodectus*) are broadly distributed worldwide, and bites from these spiders can prove fatal to humans. Venom from black widow spiders contains several well-characterized toxic proteins, the latrotoxins, different forms of which show preferential toxicity toward specific phyla of animals. Although venom toxins have been well-characterized, other tissues of this common spider have not been investigated. As part of an investigation of the biosynthesis of venom toxins, we investigated the biological properties of hemolymph and an aqueous extract of ova from *L. tredecimguttatus*. Abdomina from spiders were homogenized in water and centrifuged to pellet insoluble materials; the soluble supernatant contained hemolymph fluids. A similar method was used to extract water-soluble materials from ova. Supernatants were lyophilized and reconstituted in 0.9% saline as needed. SDS-PAGE analysis of venom, hemolymph and ova extract indicated both shared and unique components. In frog neuromuscular preparations, ova extract induced an increase (>1,000-fold) in frequency of miniature end plate potentials (MEPPS) more rapidly than crude venom, and treatment with hemolymph also resulted in increased MEPPS (with a slower onset); muscle membrane potentials remained unchanged. Ova extract produced direct hemolysis of washed human and other mammalian erythrocytes. Hemorrhage and edema were observed in rabbits at doses as low as 0.4 mg/kg ova extract. Results indicate that these tissue extracts may also contain latrotoxin-like components, and ova extracts contain phospholipase and protease activities. The functional significance of these activities in hemolymph and ova extracts is not yet clear, but their presence indicates a commonality of biosynthesis mechanisms between these tissues and venom gland epithelium.

CHARACTERIZATION OF TOXINS FROM THE VENOM OF THE BRAZILIAN SCORPION *Tityus costatus*.

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Scorpion venoms have several structurally distinct families of peptides, which are either blockers or modulators of ion channel activity. Peptides specific for: Na^+-, K^+-, Ca^{2+}- and Cl^--channels have been described. For the Brazilian scorpions the best studied venoms are those from *Tityus serrulatus*, *T. stigmurus* and *T. bahiensis*. From the venom of *Tityus costatus*, to the best of our knowledge, there is no biochemical data reported in the literature. However, recently in the area of Sao Paulo, some medical problems of envenomation due to this species were reported. In this work, we present the characterization of several toxic peptides purified from the venom of *T. costatus*, using chromatographic procedures, automatic Edman degradation, mass spectrometry and electrophysiological assays. More than fifty distinct components were obtained in pure form. Peptides with the amino acid sequence such as KEGYAMDHEGCKFSCFIGPSGFCD..., specific for Na^+-channels and a peptide whose N-terminal amino acid sequence is WCSTCLDLECGASRECYDPC FKAFGRA..., specific for K^+-channels were identified in this venom. The latter was assayed in Sf9 cells expressing the ShakerB K^+-channels. The soluble venom, as well as pure components were toxic or lethal to mice, at the doses in the range of 20 micrograms per 20 gram animal weight. These results confirm the possibility of human intoxication by the sting of *Tityus costatus* scorpion.

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ISOLATION AND CHARACTERIZATION OF HEMOLYSINS FROM *Hyla albopunctata* SKIN SECRETION.

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The presence of antimicrobial defensive agents in the amphibian skin has been suggested more than 20 years ago. The most common antimicrobial peptides belong to the following classes: magainins, bombinins, dermaseptins, brevinins, caerins and caeridins with broad antimicrobial activity. In the present report we describe the purification and partial characterization of hemolysins isolated from *Hyla albopunctata* cutaneous secretion. The animals were collected in Distrito Federal region and the secretion was obtained by electrical stimulation method and freeze-dried. Aliquots of dried secretion were submitted to a reversed-phase chromatography on a Shimpack CLC-C_8 (Shimadzu Co.) column. Eluted fractions were tested for hemolytic activity and the fractions that displayed hemolytic activity were rechromatographed using a C_{18} reversed-phase column (Vydac, 218TP54, The Separations Group). Some purified fractions were submitted to automated Edman degradation using an ABI 477A. These partial sequences were analyzed by the program BLASTP and no significant sequence homology was detected.

MOLECULAR CHARACTERIZATION OF THE INSETICIDAL NEUROTOXIN J-ACTX-Hv1c.

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New methods of insect control are urgently required due to the evolution of insect resistance to classical chemical pesticides and growing appreciation of the environmental and human health risks posed by many agrochemicals. The Janus-faced atracotoxins from the venom of the Australian funnel-web spider *Hadronyche versuta* are a family of insect-selective neurotoxins that have potential for development as a biopesticide[1].

To elucidate the molecular target of the J-atracotoxins we have developed an unbiased genetic screen in *Drosophila*. We have generated transgenic *Drosophila* that express a gene for one of these toxins, J-ACTX-Hv1c, under the control of the Hsp70 heat-shock promoter. A 10-min heat shock from 18 to 37°C is sufficient to kill transgenic flies. Mating transgenic females with EMS-mutagenized males, followed by heat shock of the F1 progeny, should result in survivors having a resistance mutation that can be mapped to reveal putative toxin target gene(s).

In parallel with the genetic screen, we have developed an *Escherichia coli* expression system for production of recombinant toxin so that structure-function relationships can be elucidated using site-directed mutagenesis. We will present a complete alanine-scanning mutagenesis of the β-hairpin region, as well as data showing that the N-terminus is important for toxin function whereas C-terminal residues are dispensible.

1. Wang *et al.* (2000) *Nature Structural Biology* 7, 505-513.

REGIONAL VARIATION OF TOXICITY IN POPULATION OF *Brachycephalus ephippium* (BRACHYCEPHALIDAE:ANURA).

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Specimens of *B. ephippium* were collected surrounding Atibaia and Mogi das Cruzes (São Paulo State), and Teresopolis (Rio de Janeiro State), all regions of Brazilian Atlantic Forest. Whole body was homogenized in MeOH/acetic acid 4%, and stored at 4° C. The extracts were evaporated, dissolved in deionized water, and the lethal potencies estimated by i.p. injection in white mice (19-20g). One mouse unit (MU, 0.22ug of TTX) was standardized as the amount of extract that kills the animal after 30 minutes of injection. The extracts were semipurificated using an exchange ionic column (Amberlite CG-50), treated with active charcoal (Norit-A), and analyzed by HPLC on a reverse phase column with post column fluorimetric reaction system. The most toxic specimens were those from Atibaia (203.63 MU/g), followed by those from Mogi das Cruzes (111.80 MU/g) and then those from Teresopolis (78.30 MU/g). The chromatographic profiles showed a quantitative difference that explains the variance in toxicity. HPLC analysis demonstrated the occurrence of TTX, tetrodonic acid, 4,9 anhydroTTX and 4-epiTTX. Twelve peaks were observed in chromatograms, which showed that TTX is not the main component in the extracts.
Finantial support: UnB and FAPDF.

PROLONGED CARDIAC XENOGRAFT SURVIVAL IN GUINEA PIG-TO-RAT AND PIG-TO-MONKEY MODELS BY A HIGHLY ACTIVE ANTICOMPLEMENTARY PROTEIN FROM THE VENOM OF *Naja kaouthia.*

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A highly active anticomplementary protein (cobra venom factor, CVF) was isolated from the venom of *Naja kaouthia* in South Yunnan, China. It displays strong anticomplementary activities in vitro and in vivo, and has 1515 U of anticomplementary activity per mg protein. The CVF was homogeneous on SDS-PAGE with a molecular weight of 149,000. It is composed of three polypeptide chains which are connected by disulfide bonds. The molecular weights of the three chains were determined to be 65,400 (α-chain), 52,100 (β-chain), and 35.500 (γ-chain). The isoelectric point of this CVF was 6.2. Its amino acid composition, carbohydrate were analyzed and N-terminal amino acid sequence of each polypeptide chain was determined. A single dose of 0.1 mg/kg CVF given i.v. to rats completely abrogated complement activity for nearly 5 days, 0.02 mg/kg CVF reduced rats' hemolytic complement activity more than 96.5 % within 6 hr and reduced monkeys' hemolytic complement activity more than 99 % within 24 hr. Its effect on prolongation cardiac xenograft survival was investigated in guinea pig-to-rat and pig-to-monkey models. In guinea pig-to-rat model, the cardiac xenograft survival time was significantly prolonged to 56.13 ± 6.27 hr in the rats treated with a single i.v. injection of 0.05 mg/kg CVF, compared with 0.19 ± 0.07 hr of control. In pig-to-monkey model, heterotopic xenogeneic heart transplantation in the abdominal cavity was performed using piglets as donors. Nine monkeys (*macaca mulatta*) were used as recipients and were immunosuppressed with a combination of cyclosporine, cyclophosphamide and steroids. Four of the recipients received CVF therapy, and complete complement depletion was achieved and sustained with CVF (0.05mg/kg, i.v. on days –3, –2, and 0.02 mg/kg, i.v. on day –0.5. After transplantation, a dose of 0.02 mg/kg per 36 hr was given intravenously). In control group, five monkeys were not treated with CVF. All recipients underwent regular blood sampling for anti-pig endotheliocyte xenoantibody and CH50, C3 and C4 levels. After rejected, the grafts were examined immunohistochemically for C3, C4, C5-9, IgG, and IgM. The results showed that in 4 recipients which received CVF therapy, complement C3 was completely depleted and no side effect due to CVF was shown, the pig hearts survival ranged from 8 to 13 days, with an average survival of 11 days. The grafts showed histopathologic features of delayed xenograft rejection (DXR). But in control group, 3 of 5 pig hearts were hyperacutely rejected after 15 to 60 minutes, the others survived for 6 days and 20 hr, respectively. Some features of HAR and DXR were described. In brief, CVF from the venom of *Naja kaouthia* showed excellent effect in overcoming hyperacute rejection and prolonged xenograft survival remarkably with rather low dosage.

CHARACTERIZATION OF THREE SHORT-CHAIN NEUROTOXINS FROM THE VENOMS OF *Naja kaouthia* DISTRIBUTED IN THE SOUTH AND SOUTHWEST OF YUNNAN CHINA.

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Three short-chain neurotoxins named NT-I, NT-II (PIR: A59136; BTR: 5052) and NT-III, were purified from the venom of *Naja kaouthia* distributed in South Yunnan province, China by serious of chromatography including SP-sephadex C25, FPLC Resource S column and HPLC reverse C18. Their molecular weight were 6952.19, 6854.92 and 6828.80 respectively by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. NT-I consisted 62 amino acid residues and the other two consisted 61 amino acid residues including 8 cysteine residues. After hydrolysis by endoproteinase Glu-C, their primary sequences were determined. Test of their activities on inhibition of muscle contractions induced by electric stimulating the rat diaphragm-nervus phrenicus preparation showed the neurotoxins blocked the binding of Ach to their receptor. The IC_{50} were 0.04ug/ml, 0.20ug/ml and 0.23ug/ml respectively for NT-I, NT-II and NT-III. The difference of IC_{50} may result by the amino acid residue substitution around Trp28, 29.

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THE EFFECT OF ERISTOSTATIN ON THE BINDING OF ANTI-α_4 TO MELANOMA CELLS.

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The disintegrin eristostatin has been shown to inhibit experimental melanoma metastasis in a mouse model. While the receptor for eristostatin is unknown, eristostatin may inhibit melanoma cell mestastasis by binding to the integrin $\alpha_4\beta_1$. Expression of $\alpha_4\beta_1$ increases in metastatic melanoma cells, and antibodies to α_4 have been shown to inhibit melanoma cell metastasis. In this study, we examined the effect of disintegrins on the binding on anti-α_4 to melanoma cells. We expressed bacterial recombinant eristostatin, echistatin, and seven mutations targeting amino acids in the RGD loop. Human MV3, 1205 LU, and WM164 melanoma cells were incubated with fluorescent-labeled anti-α_4 and an unlabeled disintegrin and were observed by confocal microscopy. Eristostatin was able to inhibit the binding of anti-α_4 to the three cell types, while EcR22V/D27W/M28L was able to inhibit the binding of anti-α_4 to MV3 and 1205LU. Truncated eristostatin inhibited anti-α_4 from binding to 1205LU and MW164 cells. Eristostatin inhibited binding of anti-α_4 to WM164 cells when asparagine at position 31 was replaced with a methionine. Echistatin was unable to inhibit binding of anti-α_4 to the three melanoma cell types. These findings suggest that the RGD motif of eristostatin is required to prevent anti-α_4 from binding to MV3 cells, and that the asparagine at position 31 is necessary for eristostatin to prevent the binding of anti-α_4 to 1205LU cells. Finally, the RGD sequence and the tryptophan at position 30 are critical for eristostatin to prevent the binding of anti-α_4 to WM164 cells.

STUDY OF TRANSFUSION ON SNAKEBITE TREATMENT.

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Large quantity of transfusion has been long used in snakebite treatment based on the concept that over-transfusion will accelerate the elimination of toxins. However, our clinical case survey and animal experiments indicated that this practices commonly used in clinic is not well scientifically adjusted. Forty-six death cases of snakebites we reviewed showed that the symptom of hemorrhagic shock, heart failure and pneumochysis are correlated with excessive transfusion. In order to prove the correlation of over-transfusion with the increased death rate, we systematically investigated response of different animals that are injected with venom. Experiments on mice, rabbits and dogs indicated that large quantity of transfusion would result in serious symptom of bleeding due to rapture of blood vessel. Moderate transfusion combined with fresh blood intermittently at early stage obviously decreased the death rate of snakebite. In summary, our results demonstrate that the traditional over-transfusion treatment on snakebite needs to be rectified.

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A PUTATIVE ANTIMICROBIAL PEPTIDE FROM THE SKIN OF *Leptodactylus labyrinthicus.*

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Amphibian cutaneous secretion has several components and among them biologically active peptides involved in several processes, including passive defense against predators and microorganisms. In the present report we describe the purification of a putative antimicrobial peptide isolated from *Leptodactylus labyrinthicus* cutaneous secretion. The animals were collected in Distrito Federal region and kept in captivity. Cutaneous secretion were obtained by mild electrical stimulation and immediately lyophilized. Aliquots of dried secretion were chromatographed on a Shimpack CLC-C_8 reversed-phase column. The hemolytic activity of eluted fractions was determined and bioactive fractions were injected onto a Vydac 218TP54 C_{18} reversed-phase column. One hemolytic peptide was purified to homogeneity and its amino acid sequence was determined by automated Edman degradation using an ABI 477A sequencer. This sequence revealed high homology to amphibian antimicrobial peptides included in the brevinin/esculentin/gaegurin/rugosin family after a search using non-redundant database by BLASTP. This proposed sequence was confirmed by electrospray mass spectrometry.

CLONING AND EXPRESSION OF INSULARIN, A DISINTEGRIN FROM *Bothrops insularis* VENOM.

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Disintegrins are proteins found in Viper venoms. They present an RGD motif which is responsible for binding to integrins from platelets and other cell surfaces. During the generation of abundant Expressed Sequence Tags (ESTs) from *B. insularis* venom gland cDNA library, constructed in pGEM11Zf^{+} vector, we found a cDNA coding for a metaloproteinase/disintegrin molecule. The encoded disintegrin sequence, termed insularin, possesses 73 amino acid residues, including 12 cysteines. This DNA fragment was subcloned in BamHI and EcoRI sites from pGEX vector, after PCR amplification. The final construct was transformed in *E. coli* BL21 and the recombinant protein, including a N-terminal fused glutathione S-transferase (GST), was obtained after IPTG induction for 3h. The fusion protein was purified by Glutathione-Sepharose 4B and analyzed by SDS-PAGE, showing an expected band of 34 kDa corresponding to GST-insularin fusion protein. This band was immunoreactive with anti-*B. jararaca* serum in a Western-Blot assay, indicating cross-reactivity with disintegrins from *B. jararaca* venom. The recombinant protein was able to bind to platelets, in a Dot-Blot test, suggesting its interaction with integrins.
The cloning of a new RGD disintegrin is a result of an EST approach aiming to characterize new transcripts from *B. insularis* venom glands. The obtaining of recombinant insularin would be significant to further studies of its role in the binding with cellular receptors.
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